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Relative Biological Activity of Some Adrenal Cortical Steroids as Determined by Eosinopenic Test.* (20522)

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Previous studies have demonstrated that any stress which will stimulate adrenal cortical function will cause a fall in the number of circulating polymorphonuclear eosinophilic leukocytes(1,2). Recently, this response of the circulating eosinophils to adrenal cortical stimulation was developed by Speirs and Meyer into a quantitative and specific method for estimating relatively small amounts of corticoids(3-5). The present paper describes the biological activity of various steroids as determined by the Speirs-Meyer test. Several points of technic are also discussed.

Methods. F_1 hybrids of C_{57} Brown female and C_{57} Black male mice were adrenalectomized and implanted with 11-desoxycorticosterone acetate (D.C.A.). The mice were housed in metal cages, and were used for the first time in the assay after an interval of at least 4 days after the operation. On the morning of the test they were injected subcutaneously with 20 μg of epinephrine hydrochloride. Four hours later, a sample of tail blood was taken, immediately after which the test sample was injected. Three hours later another sample of tail blood was drawn. Eosinophil counts of the blood were made in the Fuchs-Rosenthal or in the Speirs-Levy hemocytometer, and the percent fall in the eosinophil count in second sample relative to the first was calculated. The mean percent fall in a group of about 6 mice was used as a measure of the activity of the substance tested. Other details of the method are supplied in the paper of Speirs and Meyer(3-5). Of the first 98 mice who were adrenalectomized and pre-treated with epinephrine only 36 had counts of more than 30 cells per 2 chambers of the Fuchs-Rosenthal hemocytometer, that is, more than 93 cells per cubic millimeter of blood. Inasmuch as Speirs' suggestion of discarding such "low count" mice was followed(5), this meant that a large proportion of mice could not be used in the test. The solution of the *problem of the "low count"* mice was found to depend in part on how much D.C.A. was implanted subcutaneously at the time of adrenalectomy. At first,

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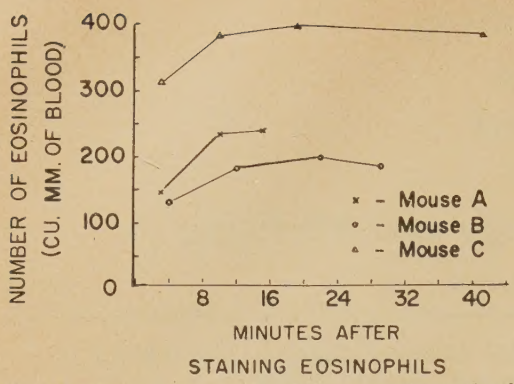
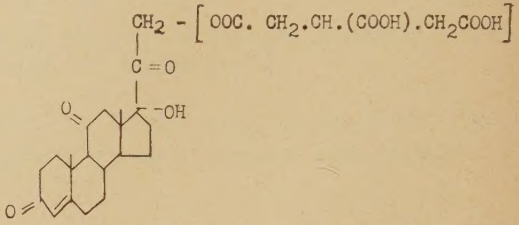


FIG. 1. Influence on eosinophil count of length of time cells were in contact with the diluent.

when a 2½ mg pellet was implanted into each mouse, the rather large proportion of "low count" mice just mentioned was the result. However, when a 5 mg pellet was used 62 out of the next 97 mice utilized had more than 93 eosinophils per cu millimeter. In these experiments, 2-3 month old mice were used. Recently, 5-6 month old mice were used, and the incidence of low count mice was further reduced to about 10% of mice adrenalectomized and implanted with 5 mg D.C.A. The mouse *blood in the Speirs-Myer* test must be in contact with the diluent for at least 10 minutes before stabilized counts can be obtained. Counts taken only 3 to 4 minutes after diluting blood were consistently lower than those counted about 10 minutes later (Fig. 1). The diluent used was that of Speirs and Meyer(3).

Corticosterone (Kendall's Compound B), 11-dehydrocorticosterone acetate (Kendall's Compound A acetate), and 11-desoxy-17-hydroxycorticosterone (Reichstein's Substance S) were tested for activity and compared with that of 11-dehydro-17-hydroxycorticosterone (Kendall's Compound E). All compounds were administered subcutaneously in 0.10-0.25 cc of 10% ethanol solution and tested at several dose levels. Sodium cholate was also tested at 2 dose levels in a few animals. In another experiment, the eosinopenic activity of 11-dehydro-17-hydroxycorticosterone-21-aldehyde (Compound E aldehyde) and of 11-dehydro-17-hydroxycorticosterone-tricarballylate (Compound E tricarballylate) were compared with that of 17-hydroxycorticosterone (Kendall's Compound F). Compound F was administered in solution containing not more than 0.75% methanol. Compound E aldehyde was injected as an aqueous solution containing 15 mg % or less of dextrose, while Compound E tricarballylate was given as an aqueous solution containing not more than 48 mg % mannitol. The structural formula of Compound E tricarballylate is



The 3 compounds were administered via the tail vein at several dosage levels but in the same injection volume—0.20 cc. In this experiment the doses were selected so that the calculations for the factorial analysis according to Bliss and Marks could be carried out (6). The *relative potencies* of Compound E, Compound A acetate, and Compound B, and of Compound F, Compound E aldehyde, and Compound E tricarballylate were estimated by Irwin's method(7). The departure from parallelism of the regression lines of the above steroids was tested by a χ^2 test.

The data of *Compound F, Compound E aldehyde, and Compound E tricarballylate* were also submitted to a factorial analysis, as described by Bliss and Marks(6), to test for the significance of the differences between steroids, for the significance of the slope of the dosage-response curve, for departure from parallelism, for curvature of the combined curve and for opposed curvature of the separate curves. For this test, equal numbers of animals at each dosage of each compound were required. Inasmuch as some groups had more than 6—the lowest number per group—data were removed from those groups with more than this number. However, the values removed were selected so that the mean, standard error and relative potency of the effected data were only very slightly and insignificantly altered by the removal.

Results. Compounds A acetate and B were

TABLE I.
Relative Eosinopenia Activities of Some Adrenal Corticoids, and Precision of the Test.*

Compound	No. of mice	Slope (b)	Index of precision (λ)	Potency ratio	Range of potency (.95%)
Compound E	28	+ 73.67	.256	100	—
" B	61	+ 65.37	.271	11	3 to 14
" A acetate	19	+102.72	.180	12	1 to 22
" F	19	+ 64.33	.402	100	—
" E aldehyde	20	+ 92.32	.337	39	26 to 58
" E tricarballylate	28	+100.37	.337	11	8 to 16

* The potency ratios of Compounds A acetate and B were expressed in terms of Compound E while those of Compounds E aldehyde and E tricarballylate were expressed in terms of Compound F.

TABLE II. Percent Fall in Circulating Eosinophil Cells Produced by Various Steroids in Adrenalectomized Mice, Pre-treated with Epinephrine.

Compound	Amt, μg	No. of mice	% fall in eosinophils, mean ± stand. error
Kendall's Compound E	1.3	5	27 ± 8
	1.6	8	36 ± 10
	2.8	7	51 ± 6
	4.6	8	69 ± 4
Kendall's Compound A acetate	7.5	7	9 ± 3
	10.0	6	26 ± 12
	30.0	6	72 ± 7
Kendall's Compound B	4.3	7	8 ± 5
	4.5	6	1 ± 1
	9.0	7	19 ± 8
	15.0	10	34 ± 7
	22.5	6	45 ± 5
	30	7	48 ± 10
	41.7	12	66 ± 6
	65	6	87 ± 4
Reichstein's Substance S	50	4	4 ± 4
	100	7	2 ± 1
Sodium cholate	2000	2	0 ± 0
	4000	2	0 ± 0
Kendall's Compound F	1.5	6	35 ± 11
	3.0	6	56 ± 8
	6.0	7	74 ± 5
Compound E aldehyde	3.0	7	16 ± 8
	6.0	6	51 ± 11
	12.0	7	72 ± 4
Compound E tricarballylate	12.0	11	22 ± 7
	24.0	9	50 ± 10
	48.0	8	82 ± 3

found to have about 12 and 11%, respectively, the eosinopenic activity of Compound E (Table I). Substance S and sodium cholate were inactive at the dosages tested (Table II). The dosage-response curves of Compounds E, A acetate and B showed a significant positive slope ($P < 0.001$), and no significant departure from parallelism between Compounds E and A acetate, or E and B.

Compound E aldehyde and Compound E tricarballylate had 39% and 11%, respectively, the activity of Compound F (Table I). That Compound E aldehyde was less active than Compound F was apparent from the fact that 3, 6, and 12 μg, respectively, of the former were required to produce an eosinopenia comparable to that obtained with 1.5, 3, and 6 μg, respectively, of the latter, while even more, that is 12, 24, and 48 μg, respectively, of Compound E tricarballylate were necessary to produce the same degree of eosinopenia (Table II). The dose-response curves of these 3 corticoids showed no significant departure from parallelism, indicating that the range of doses was in a useful portion of the curve. In all 3 steroids there was an insignificant amount of curvature and of opposed curvature.†

Discussion. The values obtained for Com-

† The solvents in which the hormones were administered did not appear to be eosinopenically active. Thus, the 10% ethanol solution showed little or no activity when administered with Substance S or with 4.5 μg of Compound B. Similarly, little or no eosinopenia occurred when a 15 mg% dextrose solution containing 1.5 μg of Compound E aldehyde was assayed or a 48 mg% mannitol solution containing 6 μg of Compound E tricarballylate was administered. Only when the same volumes of these solvents containing larger amounts of these corticoids were administered did an eosinopenia occur. Therefore, the solvents themselves were inactive. The eosinopenic inertness of the above solvents agrees with Speirs and Meyer's observation that of a wide variety of substances, including over 60 steroids, only solutions containing certain corticoids with an oxygen at C_{11} and a double bond at C_{4-5} produce an eosinopenia in epinephrine-pretreated adrenalectomized mice.

pound E in the present study were very similar to those reported by Speirs and Meyer(4). Thus, they reported a mean percent fall in eosinophils of 81, 44, and 24, 3 hours after 6, 3, and 1 μg, respectively, of Compound E were administered. Utilizing the data obtained for Compound E in the present study (Table II), the estimated mean percent fall in eosinophils at 3 hours for 6, 3, and 1 μg of Compound E was 77, 55, and 20. Similarly, Speirs and Meyer reported a 79, 60, and 13% decline in circulating eosinophils 3 hours after the administration of 6, 3, and 1 μg, respectively, of Compound F. For the same doses of Compound F, the results in similarly treated mice in our laboratory were 74, 56, and 28%, respectively. Thus, the agreement between these results was satisfactory, especially in view of the fact that each laboratory used a different vehicle for administering these hormones and also, in the case of Compound F, a different route of administration. Thus, Speirs and Meyer injected Compound F subcutaneously, while we administered it intravenously. Therefore, a comparison of the relative eosinopenic activities of the corticoids in the present study would appear to be valid even though some were administered under the skin and others via tail vein.

Of all the compounds so far tested for their eosinopenic effect by Speirs and Meyer and by this laboratory, Compound E, Compound E acetate, and Compound F were the most active. Any alteration in the structure of these steroids has so far resulted only in loss of eosinopenic activity. Thus, saturation of the 3 α, β unsaturated ketone in Compound E to give Kendall's Compound G completely destroyed this activity(4) as did also removal of the C_{11} oxygen from Compound E (see Substance S, Table II). Removal of the 17-hydroxyl from the Compound E acetate or Compound F resulted in a considerable but not total loss of eosinopenic activity (see Compounds A acetate and B, Table II). Oxidation of the C_{21} alcohol to an aldehyde also resulted in a reduction of eosinopenic activity although less so than the other changes mentioned (see Compound E aldehyde, Table II). Finally, although the acetate ester of Compound E was as active as free Compound E(4), Compound E tricarballylate appeared to be less active (Table II) even if allowance was made for the fact that only 2/3 of the molecular weight of Compound E tricarballylate consisted of Compound E.

The order of the potency of the adrenal cortical steroids as measured by the Speirs-Meyer eosinopenic test was very similar to that found by other methods, which measure some aspect of carbohydrate metabolism (11,12) or work performance(13); that is, Compounds E and F were more active than Compounds A, B and Substance S. Furthermore, Compounds A and B were found to have the same order of biological activity, whether investigated by an "eosinopenia" test (Table I), or by a "sugar activity" test (11,12,14,15). Also, Substance S showed little or no biological activity by the Speirs-Meyer test (Table II), by the "sugar activity" or "work performance" assays(16,17). Finally, while in the eosinopenia test, E was as active as F(4,Table II), or even more active(18), F had the greater efficacy in assays which measure sugar activity(11,13) or work performance(19), and in tests which measured a variety of metabolic and morphologic criteria(20).

The indices of precision, λ, were higher for the Speirs-Meyer test than for many other bio-assays of adrenal cortical steroids (Table I) but it had the advantage of being able to measure smaller amounts of the sugar-active corticoids than most other corticoid bio-assays(8). The importance of this fact was observed in a recent study which revealed that rat adrenals produce corticoids *in vitro*, and that corticotropin, added *in vitro*, stimulated this production(9,10). The amount of corticoids produced by the rat adrenals *in vitro* was too low to be detected by most of the other bio-assays but was within the range of the Speirs-Meyer method.

Summary. The eosinopenia test of Speirs and Meyer for determining small amounts of adrenal cortical steroids was found to yield dependable results in our laboratory. A series of corticoids examined for their eosinopenic effect had the following order of activ-

ity: Compound E and equally-active Compound F were most efficacious in reducing the number of circulating eosinophils. Compound E aldehyde was less active, while Kendall's Compound A acetate, Kendall's Compound B and Compound E tricarballylate were still less potent. Reichstein's Substance S had no eosinopenic activity even though it was administered in larger amounts than the other compounds. Except for Substance S, the other corticoids tested showed a significant positive regression of percent fall of circulating eosinophils on the logarithm of the administered dose within the limits of the dosages tested. The slopes of the linear regressions of these corticoids were also more or less similar.

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Immobilization of *Schistosoma mansoni* Miracidia by Immune Serum.* (20523)

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Immobilization of ciliates and free living flagellates by antibodies is well known(1,2). This report concerns the development of an immobilizing factor for the ciliated larval stage (miracidium) of *Schistosoma mansoni* by experimentally infected animals. This factor appears in the serum at about the same time in the course of the infection as do the capacities to form a precipitate around the cercariae (infective larval stage)(3), to agglutinate the cercariae(4), and to form a "hood" around the cercariae(5). It is recognized that these 3 reactions and the one here described may be caused by the same antibody.

Material and methods. The miracidia used in the immobilization tests were obtained from the livers of infected hamsters. A suspension of the infected liver in 0.15 *M* NaCl was prepared by maceration in the Waring Blender for 30-60 seconds; this suspension was allowed to settle in a sedimentation cone for ½ hour. The sediment was suspended

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in aged tap water in a 250 ml Erlenmyer flask, placed under a strong light source and the hatching miracidia collected with a capillary pipette.

Immune† sera were obtained from golden hamsters (*Cricetus auratus*) infected with *S. mansoni* for varying periods of time and from monkeys (*M. mulatta*) infected with *S. mansoni* and (*M. phillippinensis*) infected with *S. japonica*. Controls included normal monkey, hamster, and human sera. All blood from monkeys and hamsters was drawn by cardiac puncture. All sera were heated at 56°C for ½ hour.

Serial dilutions of the serum were made in 10 x 100 mm test tubes. Five drops of a given dilution were then added to 5 drops of a water suspension of miracidia which had been placed on a paraffin ringed microscope slide. Each preparation usually contained 5 or more miracidia. The mixture was allowed to stand for 10 minutes at room temperature, and the effect of the serum was determined with the aid of a binocular dissecting microscope (60x). Any noticeable effects on the miracidia were studied further by placing a coverslip over the slide and observing at 100x and 400x under a compound microscope.

The procedure outlined in the original report(3) was used in studies on the cercarial precipitate reaction.

Results. Serial dilutions of sera were tested for immobilizing effect, as shown in Table I.

In the higher dilutions of serum it was observed that some cilia were immobilized while others on the same organism continued to beat. A series of preparations at different concentrations of serum were then studied under a Zeiss phase contrast microscope. The

TABLE I. Effect of Normal and Immune Hamster Serum on the Miracidia of *S. mansoni*.

Animal No.	Days infected	Serum dilution 1/10	1/20	1/40	1/80	1/160
72 (inf.)	161	4	4	2	1	0
29 (nor.)	0	0	0	0	0	0

4 = Complete immobilization.
3,2,1 = Partial immobilization but some cilia remain mobile.
0 = No effect.

† Sera from infected animals are referred to as immune sera.

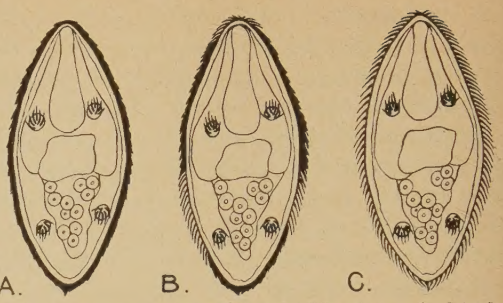

FIG. 1. Areas of immobilized cilia shown in black. A. Total immobilization (4+). B. Partial immobilization (2+). C. No immobilization (—) normal serum.

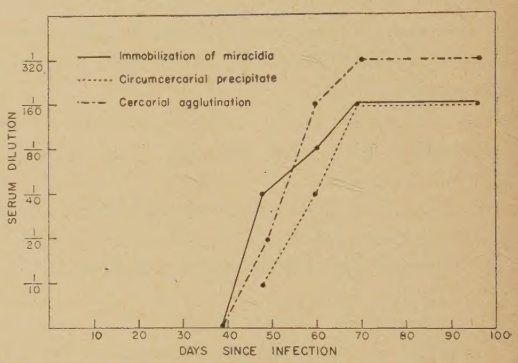

FIG. 2. Titers of *in vitro* reactions in infected monkey.

immobilized cilia were usually in the same general location on the miracidium as illustrated in Fig. 1.

Time of appearance of the immobilization antibody. A rhesus monkey infected with 500-600 *S. mansoni* cercariae was bled at approximately 10-day intervals. The sera were stored at —16°C until use. The immobilizing activity of the serum first appeared between the 39th and 48th day. It increased in titer until a maximum of 1/160 was attained at about the 75th day (Fig. 2). For comparison the titers of the cercarial precipitate reactions and the cercarial agglutination reactions are also shown. The titers of the cercarial agglutination reactions are from data previously collected by Liu and Bang(6) on these sera, but the data on the precipitate formation were obtained from new experiments.

Sera from 2 monkeys infected 5½ years previously with *S. japonica* showed a positive reaction at a dilution of 1/10. All tests utilizing normal monkey, normal ham-

ster, and normal human serum were negative, but one serum from a proven case of trichinosis in man was positive at a dilution of 1/20.

Discussion. The role of this immobilization reaction in the pathogenesis of *S. mansoni* infection is unknown. It is possible that it bears some relationship to the ray-like structures surrounding the eggs imbedded in tissue as described by Hoeppli and Li(7).

The time of appearance of the factor in the blood indicates that a possible major stimulus for its production is the initial deposition of eggs. Egg laying activity begins between the 27th and the 34th day of infection with *S. japonica*(8) and presumably during the same period in *S. mansoni* infection. The application of this reaction to studies in man remains to be made. Its possible use in the diagnosis of chronic infections is suggested by the positive reactions in monkeys infected for 5½ years.

Summary. Miracidia of *S. mansoni* were promptly immobilized when placed in heated sera of hamsters and monkeys infected with this parasite. No immobilization occurred in sera from non-infected animals. The factor developed early in the infection and was still present in the sera of monkeys which had been infected with *S. japonica* 5½ years previously. The immobilization of the miracidium appears to result from immobilization of the cilia. Cilia immobilization became less complete in high dilutions of serum and was usually confined to specific areas of the miracidium.

Since this work was done the author has become aware of an abstract by Hiyashi who reported to the Japanese Parasitology meeting in Kyoto in 1950 (Abstract 101) that he had found immobilization of miracidia from the sera of infected animals.

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Influence of Parathyroid Extract on Citric Acid of the Serum. (20524)

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It has long been recognized that the parathyroid glands are intimately concerned with the metabolism of calcium and phosphorus. In recent years, evidence has accumulated for considering the possibility that the active secretion may influence the metabolism of citrate as well. An early observation is that of Sjöström(1) who reported that increased parathyroid function produces a corresponding increase in the citric acid content of the blood. A rough parallelism has been observed between urinary excretion of calcium and of citrate in a patient with hyperparathyroidism and in a subject with hypoparathyroidism given parathyroid extract(2). Alwall(3) demonstrated concomitant increases in serum calcium and serum citric acid following intramuscular injection of parathyroid extract into dogs. The injection of parathyroid extract into puppies has been noted to increase the citrate content of bone(4). Repeated subcutaneous injections of citrate into puppies lead to osseous changes very similar to those produced by large doses of parathyroid extract(5). Freeman and Chang(6) observed, without exception, a decline in both serum calcium and serum citric acid levels following thyroparathyroidectomy in dogs. It thus appears that changes in the level of serum calcium referable to the involvement of the para-

thyroid glands are associated with parallel changes in the citrate concentration, and that the metabolism of citrate is interrelated with other physiological effects of the parathyroid hormone.

In this report are presented observations on the influence of parathyroid extract on the serum citric acid of normal and thyroparathyroidectomized rats. The work was undertaken to explore the extent of the response to varying doses of parathyroid extract and the possibility that the dose-response relationship be of such a character as to provide the basis of a method to estimate the activity of parathyroid hormone preparations.

Materials and methods. Young adult female rats of the Sprague-Dawley strain maintained on Purina rat chow pellets were used. In all experiments, involving a 9-hour experimental period or less, the animals were previously fasted for a period of 16 to 18 hours. In those instances in which the animals were studied for 18 hours, food was removed from the cages 6 to 8 hours before the experiment was begun to insure that the post-absorptive state would be attained. Thyroparathyroidectomy was carried out on fed animals approximately 24 hours prior to the experimental period. Only water was allowed to all animals during the period of observation. Studies were made employing 3 different routes of injection: subcutaneous, intraperitoneal, and intravenous via the external jugular vein. Blood samples were obtained by cardiac puncture. Parathyroid extract (Lilly)* was used as a standard preparation throughout the experiments. For doses of less than 100 U.S.P. units, the extract was diluted with 0.9% sodium chloride solution so that, with the exception of those animals receiving 200 units, the volume injected was 1.0 ml. The method of Natelson, Pincus, and Lugovoy(7) was employed for the microestimation of serum citric acid. Isooctane (Phillips) was employed for the extraction of the pentabromoacetone. The optical density readings were made with a Coleman Model 11 Universal Spectrophotometer at 445 mμ, using rectangular cuvettes with a 4.0 cm light absorption path. The cuvette holder was a shielded side carrier modified for use with the small volume of fluid available for analysis.† For the analysis of serum calcium, the micro method of Rappaport and Rappaport(8) as modified by Biering(9) was employed.

Results and discussion. The subcutaneous injection of 50, 100 and 200 units of parathyroid extract resulted in definite increases in the serum citric acid level within 18 hours after administration. The major increment of this response appears to be elicited no earlier than 9 hours subsequent to the administration of the extract. In this respect, the time-response relationship is quite similar to that reported by many investigators for serum calcium(10). The administration of 200 units was no more effective in influencing the serum citrate levels than was 100 units under these conditions. The expectation that intraperitoneal and intravenous injections might lead to more consistently positive response was not realized in exploratory trials.

Since it is a generally accepted view that relatively smaller amounts of the hormone are required to maintain normal serum calcium levels in the thyroparathyroidectomized animals than are required to obtain increases in the serum calcium level in normal animals, attention was directed to the use of operated animals. The responses of thyroparathyroidectomized female rats 18 hours after the subcutaneous injection of 25, 50, and 100 units are shown in Table I. The greatest degree of uniformity and the most sensitive response to the injected dose was found under the experimental conditions of this study. The mean initial level of serum calcium 24 hours subsequent to bilateral thyroparathyroidectomy was 7.2 mg %. This value is in agreement with that given by Tweedy and Chandler(11) in their study correlating complete parathyroid extirpation with the drop in serum calcium level.

The responses to subcutaneous injections of graded doses of the extract are similar in

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† We are grateful to Mr. Walter C. Burfischer of the Wilkens-Anderson Co. for his time and effort in the design of this carrier.

TABLE I. Effect of Graded Doses of Parathyroid Extract on Serum Calcium and Serum Citric Acid of Thyroparathyroidectomized Rat 18 Hours after Subcutaneous Injection.

Dose units	No. of observations	Serum calcium: Mean initial level (mg %)	Serum calcium: Mean* change (mg %)	Serum citric acid: Mean initial level (mg %)	Serum citric acid: Mean* response (mg %)
25	7			4.07	+1.33 ± .28
50	6	6.9	+3.2 ± .30	4.70	+3.33 ± .35
100	6	7.7	+4.3 ± .37	4.88	+2.84 ± .30

* Mean ± stand. error of the mean.

the normal and thyroparathyroidectomized rat in that there is observed in both groups a rise in serum citrate to a maximum after which the administration of larger doses does not produce an increased response. In both groups, the maximal level to which the serum citrate rises was found to be very similar. However, the dose levels at which the phenomenon occurs is different in the 2 groups of animals. Thyroparathyroidectomized rats respond maximally to the administration of only 50 units, whereas normal animals respond maximally to 100 units. It is also observed from Table I that the operated animal is sensitive to the injection of 25 units. No special significance can be assigned at this time to the differences in serum calcium at the 2-dose levels of 50 and 100 units. The mean pre-injection levels of serum citrate in all the thyroparathyroidectomized rats used in this work (4.49 mg %) is significantly lower than that observed in all normal animals (6.75 mg %). There was also observed in the operated animals less variability between individual pre-injection levels and post-injection levels at a given dose than in normal animals after the administration of the same amount of extract.

Although influences exerted by parathyroid extract injections on serum citric acid levels in both normal and thyroparathyroidectomized animals are definite, these experiments do not of themselves fully define the quantitative nature of the observed response. From dose-response data accumulated on the operated animals, a linear relationship appears likely between 0 and 50 units. However, the experiences of this study lead to the conclusion that the use of serum citrate responses in the rat would offer no advantage over existent methods in assessing the potency of parathyroid hormone preparations.

As concerns the source of the increased citrate observed following injections of parathyroid extract no satisfactory explanation can be given. The discovery by Dickens(4) of considerable stores of citrate in bone would suggest that the blood changes after parathyroid extract injections or parathyroid extirpation reflect differences in the degree to which this substance is mobilized from bone.

Summary. The subcutaneous injection of parathyroid extract into normal and thyroparathyroidectomized rats results in definite increases in the serum citric acid level. Dose-response data indicate that the rise in serum citrate varies with the dose of the extract, the route of the injection and the period of observation. The level of serum citrate of thyroparathyroidectomized rats was found to be significantly lower than that of normal animals. Increased sensitivity and greater uniformity of response to the injected dose was observed in the operated animals as compared to normal animals.

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Propagation of a Strain of Egg-Adapted Distemper Virus in Suckling Mice.* (20525)

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Several workers have adapted the canine distemper virus to growth on the chorioallantoic membrane (CAM) of the embryonating egg(1-4). Its continued passage in the egg has resulted in a gradual loss of pathogenicity for naturally susceptible species. Simultaneous retention of the antigenicity and immunogenicity of the virus have led to its use as a vaccine(3,5).

Serial passage of an egg-adapted strain of distemper virus in suckling mice was accomplished in this laboratory recently. Two lines of virus were propagated by intracerebral inoculation through 18 and 10 passages, respectively. Nervous signs of moderate degree which were apparent in a small proportion of mice of the first passage of each line became quite pronounced and prevalent with subsequent passages.

Materials and methods. The FXNO strain of egg-adapted distemper virus was used in this study. This strain, originally isolated from infected fox spleens(4), was carried through 80 egg passages in this laboratory. By the 45th passage, the virus induced small, widely scattered, opaque, papuloid areas of the CAM. Fiftieth egg passage tissues elicited only a mild infection in ferrets. After recovery these animals were immune to challenge with virulent distemper virus(6). A Rockefeller line of Swiss albino mice originating from Carworth Farms stock served as the host. Mice varying in age from 1 to 4 days were inoculated intracerebrally in the right hemisphere with 0.01 ml quantities of inoculum. Infected chorioallantoic membranes, ground and centrifuged for 10 minutes at 2500 rpm in an International centrifuge furnished the supernatant fluids used as inoculum for the initial passage. Twenty percent suspensions of infected brains in tryptone broth were centrifuged similarly and used for all subsequent passages. For titrations, 10-fold serial dilutions were made in tryptone broth. Mice with definite nervous signs were killed with ether and stored in a deep freeze at —20°C. Hyperimmune sera collected from ferrets after vaccination with the FXNO strain and subsequent challenge with virulent virus (Delavan strain) were used in virus neutralization experiments. Sera collected from ferrets which had not had previous experience with the distemper virus served for control purposes. All sera were heated at 60°C for 30 minutes just prior to use. The Delavan strain of virulent distemper virus used for challenge exposure of ferrets was obtained by Dr. John R. Gorham from a natural outbreak of distemper on a mink ranch near Delavan, Wis. Ten or 11 days after inoculation of ferrets with this strain, the skin of the abdomen and the chin became reddened. By the 13th day, a purulent exudate of the eyes was present and the eyelids became pasted together. The appetite was gradually lost; the animals became sluggish and died in 24 to 30 days. It was not uncommon for those surviving longer than 24 days to show nervous signs.

Mouse passages. Line 1, now in its 18th mouse passage, was initiated from the 65th egg passage of the FXNO strain. Line 2 originated from the 75th egg passage and is now in its 10th passage in suckling mice. The observations of the passages of both lines are summarized in Table I. Hyperkinesis was the most striking sign in the early passages of both lines. This manifestation, rapid running and jumping at the sides of the cages, persisted for 2 to 3 days at which time the mice were sacrificed for further passage of the virus. By the 8th passage ataxia, as well as hyperkinesis, was observed on the 4th day

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TABLE I. Intracerebral Passage of 2 Lines of FXNO Distemper Virus in Suckling Mice.

Passage No.	Age of mice in days		No. affected/ No. inoculated		Time in days after inoculation until appearance of nervous signs	
	Line 1	Line 2	Line 1	Line 2	Line 1	Line 2
1	1	3	2/8	1/11	8	15
2	3	4	6/6	5/9	6	13
3	2	4	2/11	4/8	7	7
4	3	3	6/7	5/6	5	5
5	3	2	9/10	6/6	4	6
6	4	3	9/9	8/8	4	5
10	4	4	9/9	5/7	3	4
18	3	—	8/8	—	3	—

after inoculation and was succeeded rapidly by muscular twitching, disequilibrium, paresis, and clonic spasms. With the 10th passage, nervous signs appeared in 3 days with Line 1 and in 4 days with Line 2. Deaths occurred within 5 to 8 days of inoculation. The LD_{50} titer/g of infected brain material gradually increased with passage. The approximate LD_{50} titers for the 5th, 10th, and 16th passages of Line 1 were 10^3, $10^{4.5}$, and 10^6, respectively.

Infectivity of mouse-passaged virus for embryonating eggs. Twenty percent suspensions of brain material from the 5th, 6th, and 11th passages, respectively, of Line 1, when ground in tryptone broth containing penicillin and streptomycin (1000 units and 1000 μg per ml, respectively), and inoculated onto the CAM of 7-day eggs, produced lesions typical of the original FXNO strain. In each case, the area of CAM affected was slight so that a repassage was necessary to produce prominent lesions.

Immunizing effect on ferrets. Seven 4-month-old ferrets not previously exposed to the distemper virus were inoculated intraperitoneally with one ml quantities of 20% suspensions in tryptone broth of the following: brains of normal mice into 2 ferrets, brains of mice infected with the 6th mouse passaged FXNO strain, Line 1, into 3 ferrets, and chorioallantoic membranes infected with the original FXNO strain into 2 ferrets. Clinical signs of infection did not develop in any of the ferrets during the subsequent 3 weeks. At this time the 7 ferrets and 2 additional ones received an injection of distemper virus of the Delavan strain. The ferrets which had received the infected chorioallantoic membranes or the infected mouse brains did not show clinical signs of disease during the 3-month observation period following challenge with the virulent Delavan virus. The ferrets previously injected with normal mouse brain as well as the untreated controls exhibited the typical clinical signs of distemper in this species. Ferret protection tests indicated that after 6 intracerebral passages in mice, Line 1 had retained the immunogenicity of the original egg-adapted virus for ferrets against clinical distemper. Since the virus titer of the fluid expressed from chorioallantoic membranes infected with the FXNO strain is approximately 10^3 to 10^4 ID_{50} per ml, it is not likely that a sufficient quantity of the original virus may have been carried through 6 passages in mice to bring about the protection indicated.

Neutralization tests. Attempts to evaluate the neutralizing activity of the ferret sera by intracerebral inoculation of the serum-virus mixtures in suckling mice have yielded equivocal results. However, a modification of the technic of Belcher(6) employing the CAM of 7-day embryonating eggs as the test tissue has given definite results. A tryptone broth suspension of virus from the 11th mouse passage, after 2 subsequent passages in eggs, was used as inoculum. This suspension contained approximately 1000 ID_{50} per ml. Immune serum dilutions of 1-4 to 1-64 were admixed with equal aliquots of inoculum and 0.2 ml quantities injected into 4 eggs per dilution. A mixture of virus and non-immune ferret serum was employed as a control for the experiment. The results are shown in Table II.

The hyperimmune serum pool completely inhibited the formation of lesions of the CAM

TABLE II. *In ovo* Neutralization of 100 Embryo ID_{50} of Mouse-Brain-Propagated, Distemper Virus by Distemper Immune Serum.

Final serum dilutions	Normal serum	Immune serum
1-8	4/4*	0/4
1-16	"	"
1-32	"	"
1-64	—	1/4
1-128	—	"

* No. egg membranes showing lesions/No. inoc.

in dilutions as high as 1-32 while normal serum had no observable neutralizing effect.

Summary. Two separate lines of the FXNO strain of egg-adapted canine distemper virus were passed intracerebrally in suckling mice 18 and 10 times, respectively. Both lines induced signs of protracted hyperkinesis. After the 7th passage central nervous system involvement became quite prominent and severe. Ataxia, muscular twitching, disequilibrium, paresis, and clonic spasms were common manifestations. The time of the first nervous disturbance decreased progressively from 8 days to 3 days in Line 1 and from 15 days to 4 days in Line 2. *In ovo* neutralization tests and ferret protection tests establish the identity of the mouse passaged agent with the original egg-adapted FXNO strain.

Comment. While this manuscript was in preparation, a Lederle strain of avianized distemper virus (Lederle Distemper Vaccine Mink Serial No. 6368-17A) was passaged 6 times in suckling mice with results in agreement with those reported for the FXNO strain. This would suggest the lack of strain specificity in the mouse adaptability of the avianized distemper virus.

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Infrared Spectrophotometry as a Means for Identification of Mosquitoes.* (20526)

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In addition to the conventional taxonomic methods, various types of differentiating procedures have been employed in attempts to distinguish between closely related species of mosquitoes. The *Culex pipiens* complex in this country, for example, has been studied through cross-breeding experiments, temperature responses, susceptibility to parasitic infections as well as immunologically(1). Such investigations have been made in the search for distinguishing characteristics whereby the exact relationship between morphologically similar species might be determined. These biological tools, however, are very time-consuming and might well be supplemented by other methods. Until recently, no attempt was made to employ biochemical procedures as a means of comparing mosquito species. The first step in this direction was made by Micks and Ellis(2) in which the free amino acids of 7 species of mosquitoes were analyzed by paper chromatography. Certain quantitative differences were found between the

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various genera of mosquitoes used, while such differences between the species of a single genus were less marked.

In searching for a more precise method for comparing biochemical constituents, it was decided to use infrared spectrophotometry. The use of this method for the analysis of proteins and complex biological substances has been extended in recent years(3-11). Concomitant with this development, the potential utility of infrared spectra for the identification of naturally occurring substances has been indicated, notably among which has been the identification of bacteria(7,11). This report represents the initial studies in the application of this technic to the identification of mosquito extracts.

Materials and methods. The mosquitoes used were *Culex quinquefasciatus, Culex molestus* (autogenous) and *Aedes aegypti,* all of which were maintained as laboratory colonies. The larvae were fed a standard diet of finely powdered dog chow. Unfed, adult female mosquitoes were allowed to emerge in lantern chimneys and all batches were approximately the same age when used (24-48 hours). They were anesthetized with chloroform and immediately weighed, after which they were transferred to a tissue grinder and homogenized in distilled water (2 ml per 125 mg of mosquitoes). The material was centrifuged at 5000 RPM for 15 minutes and the supernatant retained. This procedure was repeated twice, resulting in a supernate free of visible suspended material. Two tenths of a milliliter of the aqueous extract was spread evenly over the surface of a silver chloride plate. The material was applied in the shape of the aperture of the liquid micro-cell which was adapted to hold a one-inch square silver chloride plate. The area of the preparation was slightly larger than the aperture so that the sample completely filled the optical path. The specimens were dried slowly at 37°C and analyzed within 24 hours after preparation. The infrared absorption spectra from 2-16 μ were determined with a Baird double-beam recording instrument equipped with a sodium chloride prism.

Results. It was necessary to establish the reproducibility of the method used for the preparation of the extracts. Fig. 1 presents the spectra of 4 different batches of *C. molestus* extracts. These spectra were found to be qualitatively reproducible, thus making it possible to detect the subtle differences that existed among extracts of the different species of mosquitoes studied. Although it has not been possible to reproduce with complete accuracy the quantity of absorbing material which probably accounted for the variation in the amount of light scattering, it would appear that this difficulty is also inherent in various other complex systems. This has been reported by Stevenson and Bolduan(7) with bacteria and observed by Benedict and Pollard(12) with virus preparations. Nevertheless, the quality of the films was judged by the same criteria as reported by Stevenson and Bolduan(7) namely, the use of the maximum transmission values at 5.5 and the minimum value at 6.1 μ.

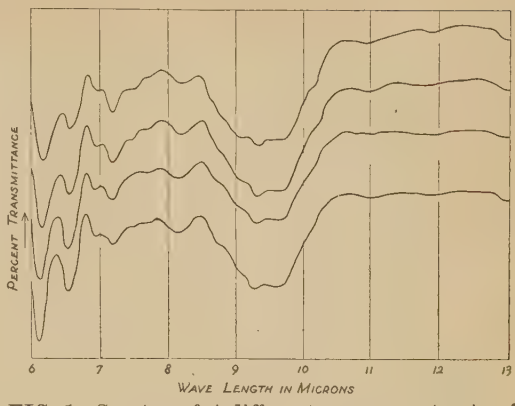

FIG. 1. Spectra of 4 different aqueous extracts of *Culex molestus* mosquitoes

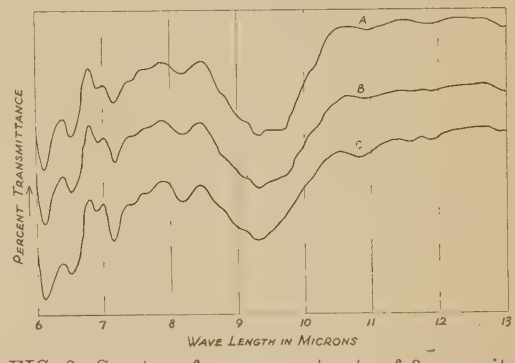

FIG. 2. Spectra of aqueous extracts of 3 mosquito species: A, *Culex molestus*; B, *Aedes aegypti* and C, *Culex quinquefasciatus.*

Fig. 2 shows the spectra of aqueous extracts of *C. molestus*, *C. quinquefasciatus*, and *A. aegypti* within the most informative range (6-13 μ). Although the same major absorption bands were observed, sufficiently consistent differences made possible the identification of these mosquitoes. The most important differences were the relative intensities of the peaks at about 6.95 and 7.2 μ, and the relative depth and shape of the broad band from about 8.6 to 10 μ. In one instance, a preparation was made using *C. molestus* males and the resulting absorption bands were indistinguishable from those of the females of this species.

Six weeks storage of the smeared silver chloride plates at room temperature rendered certain changes in the spectra. There was a loss of considerable definition of the band from 8.5 to 10 μ.

Discussion. The data given in this report indicate that infrared spectrophotometry is a simple and rapid method for the identification of the mosquito extracts studied. Moreover, sufficient differences existed among the species used to indicate that specific identification by this method is possible. It is interesting to note that the biochemical identity of mosquitoes may not follow the classical taxonomic pattern. For example, the extracts of *C. molestus* demonstrated absorption bands more similar to those of *A. aegypti* than those from a mosquito of the same genus (*C. quinquefasciatus*). It seems obvious, however, that no conclusions can be drawn at the present time regarding this point.

The characteristics of the spectra were not a function of concentration since it was observed that by titrating *C. molestus* and *C. quinquefasciatus* extracts the qualitative shapes of the curves did not change although the intensities of the bands decreased proportionately with dilution. The crude aqueous extracts were satisfactory for analysis, however, it may well be that further processing will yield preparations exhibiting even greater differences, thereby facilitating the infrared analysis. The utility of this method for the identification of other genera and species of mosquitoes is being investigated and will be the subject of another report.

Summary. Crude aqueous extracts of *Culex molestus, Culex quinquefasciatus,* and *Aedes aegypti* adult mosquitoes were identified by infrared spectrophotometry. The most important differences were the relative intensities of the peaks at about 6.95 and 7.2 μ, and the relative depth and shape of the broad band from about 8.6 to 10 μ.

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Renal Response to Hypercapnia.* (20527)

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The role of the kidney in aiding in the readjustment of disturbances in acid-base balance created by respiratory acidosis has until recently received little attention(1-3). In diffusion respiration, characterized by progressive increase in blood CO_2 concentration and decreased blood pH, significant and sometimes profound alterations in renal function occur. Among these are oliguria and decreased urinary pH(4-8). Hypercapnia also induces a decrease in sodium, chloride, bicarbonate, and potassium excretion with simultaneous increase in the excretion of ammonia and phosphate(1-4,9).

The inference has been made that the oliguria results from direct or indirect action of CO_2 on the renal vasculature, presumably causing reduction of glomerular filtration rate(5,9,10). The question, therefore, arises as to whether the alterations in electrolyte excretion are due to direct tubular action or are actually due to changes in glomerular filtration rate which may accompany respiratory acidosis. It therefore appeared expedient to investigate the renal circulatory changes which occurred under conditions of progressive hypercapnia. This was done with the use of standard renal clearance procedures in dogs. Concomitant observations were made on blood CO_2 content, urinary pH, urinary volume, and excretion of sodium and potassium.

Methods. Experiments were conducted under sodium pentobarbital anesthesia (30 mg/kg, given intravenously). Following necessary surgical procedures, an intravenous sustaining solution containing creatinine, PAH, and mannitol, the last as a 10% solution, was delivered at a rate of 1.2 to 2 ml/min. Suitable priming doses were given initially. Urine was collected for 2 consecutive 10-minute periods during a control stage, 4 stages during rebreathing, and 2 recovery stages. A 20-minute discard period preceded each stage. Arterial blood samples were collected at 30-minute intervals throughout the experiment beginning in the first urine collection period. Plasma concentrations used were obtained by interpolation of the plotted data. In order to produce a progressive hypercapnia, the dogs were rebreathed from a segment spirometer (11) which had been flushed with pure O_2 to prevent hypoxia. This was attached to a tracheal cannula immediately following the second control urine collection. The animal respired from the spirometer for 20 minutes before the experimental urine samples were collected. With continued rebreathing the CO_2 content of the spirometer gas was progressively elevated during the 4 stages of hypercapnia. The spirometer was then disconnected and the animal allowed to breathe room air for 20 minutes before the recovery stages were observed. Six consecutive analyses of CO_2 and O_2 were made on spirometer gas during the rebreathing, using a Henderson-Morris apparatus(12). The CO_2 content of the arterial blood was determined in 6 of the experiments with the Van Slyke apparatus(13) on samples drawn during the control, second, fourth, and recovery stages. Hematocrit determinations were made on alternate blood samples. Urine and plasma sodium and potassium were measured with an internal standard flame photometer. Urine pH was determined on each sample with a Leeds and Northrup pH meter. The alkaline picrate method was used to analyze the plasma sodium tungstate filtrates and the urine for creatinine. The method of Smith(14) was used for the PAH analysis of urine and the plasma cadium sulfate filtrates.

Results. The results were obtained on 8 animals, but for technical reasons all measurements were not made in every experiment.

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† An investigation conducted in partial fulfillment of the requirements Phase I of the Medical Curriculum of Western Reserve University School of Medicine.

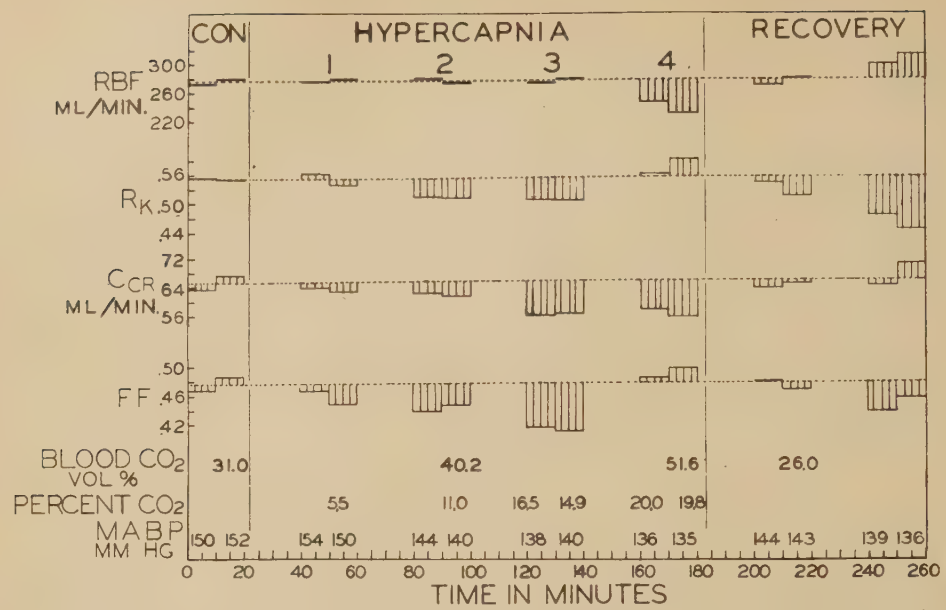

FIG. 1. Influence of progressive hypercapnia on renal vasomotor function. RBF: renal blood flow based on PAH clearance; R_K: renal vascular resistance; C_{CR}: creatinine clearance, a measure of glomerular filtration rate; FF: ratio of C_{CR}/C_{PAH}.

Rebreathing caused the CO_2 content of the spirometer gas to increase progressively to an average of 16.8% (range 13.5-19.9) during Stage 4. Paralleling the increase in the CO_2 concentration of the inspired air was a marked increase in respiratory rate and respiratory minute volume, and a decrease in heart rate. Hyperventilation was observed to continue for a time after the return to room air; then respiration gradually became slow and shallow during the later recovery periods.

Mild hypercapnia appeared to have little effect on blood pressure; however, as hypercapnia became more severe, it decreased to a level approximately 10% below the control average of 149 mm Hg (range, 138-162). During recovery, blood pressure increased slightly but, like heart rate, did not reach control level.

To simplify the presentation of the effects of hypercapnia on renal hemodynamics, a typical experiment has been chosen for detailed consideration as representative of the over-all data (Fig. 1). At the beginning of the experiment, blood pressure was 150 mm Hg of mercury; it decreased during the second stage, when it averaged 142 mm; then finally declined to 135 mm during maximal hypercapnia. Blood pressure was restored to 144 mm on return to room air. Very little change in renal blood flow occurred until the later stages of hypercapnia; during Stage 4 it decreased from 275 ml/min. to 230 ml/min. Levels at or slightly above control values were reached during recovery. Renal resistance tended to remain constant during the first stage of hypercapnia, but decreased slightly during Stages 2 and 3. During Stage 4 there was a trend toward increased resistance which did not, however, persist during the subsequent recovery periods.

Glomerular filtration rate, as measured by creatinine clearance, exhibited a progressive slight tendency to decrease. During recovery it returned to near control levels. The filtration fraction in this experiment showed a decrease in the Stages 1, 2, and 3. However, during Stage 3, when renal resistance increased, the filtration fraction also increased.

The data on renal hemodynamics for all 8 dogs are shown in Fig. 2. The values, as ratios to the control average, are related to CO_2 content of the inspired air. The over-all effect on renal blood flow, in the range of CO_2 content of inspired air observed, did not appear to be significant.

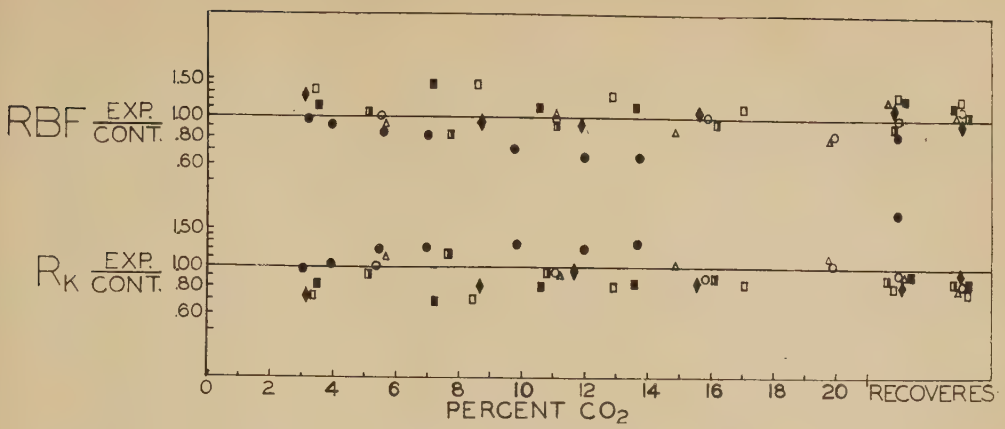

FIG. 2. Summary of RBF and R_K changes given as percentage of control, and related to the CO_2 content of the inspired air.

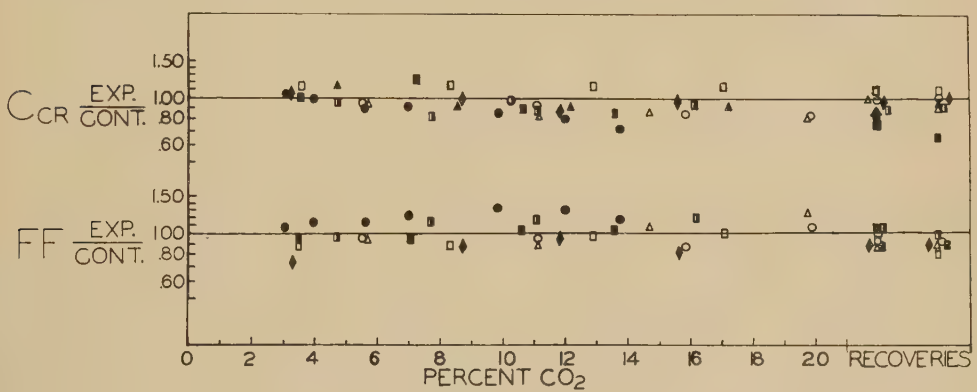

FIG. 3. Filtration rate and FF changes as related to CO_2 content of the inspired air.

The over-all trend of filtration rate is shown in Fig. 3. It can be seen that there existed a small downward trend. The filtration fraction, considering the group as a whole, revealed no consistent change.

Urinary pH declined steadily during hypercapnia to an average of 6.2 (range, 5.75-6.7) during Stage 4 from a control average of 7.3. This decrease was related to a progressive increase in arterial blood CO_2 content from 35 volume % during the control period to an average of 52.8 at the peak effect, then to 32.2 volume % during the recovery stages at which time urinary pH rose to 7.50. Plasma sodium concentration increased slightly (7.4 mEq/L) above the control average of 137 mEq/L, and plasma potassium increased 1.27 mEq/L (control average, 3.53 mEq/L) during hypercapnia.

Changes in electrolyte excretion are presented in Fig. 4 as deviations from the control average. The average control value for sodium excretion was 0.174 mEq/min. (range, 0.016 to 0.369), and for potassium 0.056 mEq/min. (range, 0.036 to 0.108). A downward course in sodium excretion is apparent in these experiments, with an average ratio to control of 0.355 in Stage 4. An interesting finding was an increase in the excretion during the recovery periods to values as high as 4.7 times the control rate in one experiment and averaging 1.12 in all experiments. It is evident from the figure that potassium excretion exhibited a similar trend. In Stage 4, excretion averaged 0.70 of control and rose to 1.94 during recovery.‡

The urine volume increased and remained consistently above the control volume throughout hypercapnia. During recovery the urine volume tended to increase to an even higher level. However, no significance can be attached to this observation since

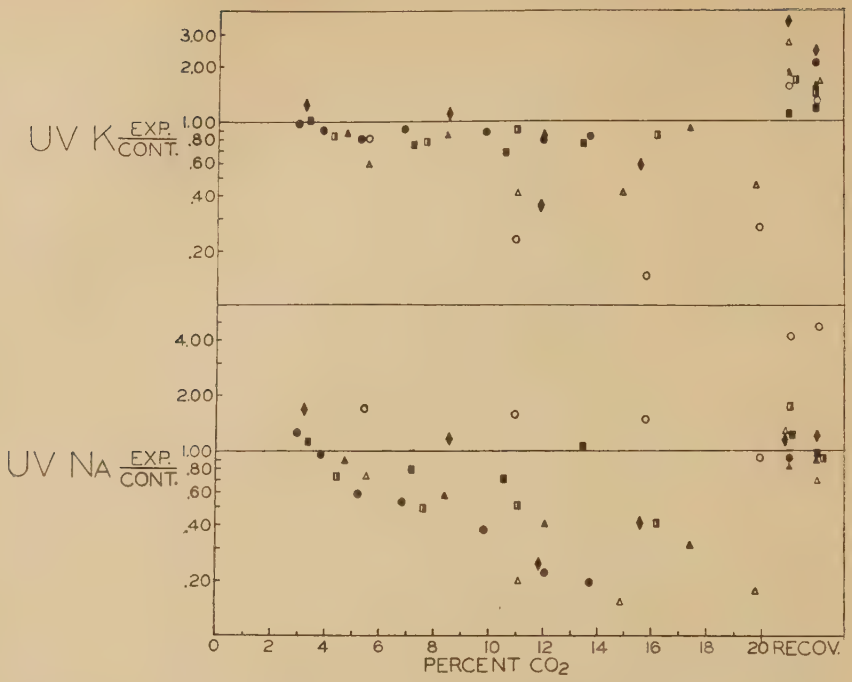

FIG. 4. Changes in electrolyte excretion resulting from hypercapnia, given as percentage of control related to % CO_2 in the inspired air.

mannitol, acting as an osmotic diuretic, was constantly infused. Its action would tend to interfere with any alterations in water reabsorption due to hypercapnia; therefore, no further consideration of the changes in urine volume can be made.

Discussion. Fig. 3 reveals that there is a tendency for filtration rate to decrease during the period of hypercapnia in all dogs but one. Although the magnitude of the decrease is too small to claim significance in individual animals, the consistency of the trend strongly suggests that the alteration is real and worthy of consideration. In this connection it has been conclusively demonstrated that small reductions in glomerular filtration rate, resulting in minimal decreases in load, can cause electrolyte excretion to diminish(15,16). Apparently, with a decreased load, sodium and potassium excretion are reduced because of more efficient reabsorption of the filtered electrolyte. One must therefore be cautious of accepting the present data as proving that the reduced electrolyte excretion is solely the result of altered renal tubular mechanisms. Nevertheless, certain of the experiments do suggest that hypercapnia has an additional direct effect upon the tubular cells which influence their reabsorptive and secretory functions. An example is presented in Table I. During hypercapnia the percentage of load reabsorbed is increased and excretion diminished in periods 8, 9, and 10 despite no decrease in load. Note that potassium excretion diminishes during periods 7 to 10 despite actual increases in load.

In another experiment (solid squares in Fig. 3 and 4) the filtration rate increased 19% at 7.2% CO_2, but the sodium excretion decreased 21%. In this experiment potassium excretion was also diminished somewhat. These findings show that in respiratory acidosis increased sodium reabsorption may be stimulated as a part of a base conserving mechanism by the kidneys, as demonstrated

‡ One experiment was atypical with regard to effects on electrolyte excretion, and was therefore not included in Fig. 4. The excretion of sodium and potassium increased in this animal and did not show a characteristic decline during hypercapnia. Perhaps bearing on this effect was the fact that filtration rate increased to 15% above the control level and remained elevated throughout the course of the experiment.

TABLE I. A Representative Experiment Showing Influence of Hypercapnia on Electrolyte Excretion.

Period	CO_2 inspired air, vol/%	Arterial CO_2, vol/%	Urinary pH	P, mEq/ml	GFR, ml/min.	Load, mEq/min.	U, mEq/ml	V, mEq/min.	UV, mEq/min.	Amt reabsorbed	% reabsorbed
					Sodium						
Control											
1		33.7	7.55	.123	41.4	5.08	.026	1.5	.039	5.04	99.3
2	.0		7.50	.124	44.3	5.50	.037	1.6	.059	5.44	98.7
Hypercapnia											
3			7.10	.127	49.3	6.26	.034	2.4	.082	6.18	98.6
4	3.3		7.22	.125	45.1	5.64	.036	2.4	.087	5.55	98.5
5			6.95	.128	45.5	5.82	.030	2.3	.069	5.75	98.7
6	8.62	43.2	6.85	.131	40.9	5.35	.023	1.9	.044	5.31	99.3
7			6.25	.132	35.7	4.72	.013	1.0	.013	4.71	99.6
8	11.93		6.14	.132	38.6	5.08	.011	1.1	.012	5.07	99.7
9			6.14	.133	42.2	5.62	.012	1.4	.017	5.60	99.7
10	15.62	55.2	6.25	.133	41.4	5.50	.016	1.5	.024	5.48	99.7
Recovery											
11		37.1	7.55	.130	47.0	6.12	.026	2.3	.060	6.06	99.2
12	.0		7.59	.130	41.0	5.32	.030	2.2	.066	5.25	98.7
13			7.70	.150	38.7	5.80	.032	1.7	.054	5.75	98.9
14	.0		7.70	.156	38.0	5.81	.037	1.7	.063	5.75	98.8
					Potassium						
Control											
1		33.7	7.55	.0040		.166	.029		.043		
2	.0		7.50	.0040		.179	.028		.044		
Hypercapnia											
3			7.10	.0041		.202	.024		.056		
4	3.3		7.22	.0042		.192	.024		.057		
5			6.95	.0048		.217	.024		.055		
6	8.62	43.2	6.85	.0050		.204	.024		.045		
7			6.25	.0054		.193	.015		.015		
8	11.93		6.14	.0054		.208	.015		.016		
9			6.14	.0057		.242	.016		.023		
10	15.62	55.2	6.25	.0060		.247	.018		.026		
Recovery											
11		37.1	7.55	.0059		.276	.070		.162		
12	.0		7.59	.0057		.233	.065		.144		
13			7.70	.0055		.212	.066		.111		
14	.0		7.70	.0055		.208	.059		.101		

for metabolic acidosis(17,18).

Concomitant with the reduction in urinary sodium and potassium excretion there is increased hydrogen ion secretion. Presumptive proof of this is the decreasing urinary pH during hypercapnia. Hydrogen ions are secreted in the tubular lumen in increased amounts because of the elevated concentration of H^+ in the blood and tubular cells. Simultaneously, the tubular cells reabsorb sodium more efficiently. The fact that potassium excretion is reduced suggests that H^+ secretion proceeds in preference to K^+ secretion in the ion exchange mechanism(19).

The increased plasma potassium concentration during hypercapnia may be the result of mobilization of this ion from intracellular compartments. Renal retention of potassium is another possibility. The slight increase in plasma sodium concentration can probably be accounted for on similar bases.

After return to room air the dogs continued to hyperventilate and apparently went into a mild degree of respiratory alkalosis. Evidence of this was the reduction of blood CO_2 to 32.2 volumes % and the increase of urinary pH to 7.5 during the recovery stages. Apparently, this reversed the processes of electrolyte excretion, for now both sodium and potassium were excreted at a more rapid rate than normally.

No final answer can be given in explanation of the oliguria observed during hypercapnia with diffusion respiration(4-8). Oliguria did not occur, and only slight reduction in glomerular filtration rate was observed during hypercapnia in the present experiments. Perhaps the phenomenon was masked by the osmotic diuretic action of the mannitol.

Summary. An average increase of 17.9 volume % in arterial blood CO_2 concentration was produced in dogs by rebreathing from a spirometer. No evidence of significant renal vasomotor alteration was noted. However, urinary pH decreased, and sodium excretion progressively decreased to an average of 35% of control. Thus, elevated H^+ concentration of the blood had in some manner enhanced the tubular reabsorption of sodium. Potassium excretion also diminished, presumably due to depressed tubular secretion by the predominance of H^+ made increasingly available for exchange with sodium. Plasma concentration of potassium increased during hypercapnia, due to renal retention or mobilization from the intracellular compartment. Plasma sodium showed a similar trend. Hyperventilation occurred for a time on return to room air. This phase manifested a decrease in blood CO_2, increase in urinary pH, and increase in the excretion of potassium and sodium beyond control averages.

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Ultrafiltrability of Plasma Urate in Man.* (20528)

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The urate clearance in normal man is of the order 7-10 cc/min. at adequate urine flows and therefore only some 5-8% of the inulin clearance. Opinion as to the significance of this urate clearance deficit is still divided(1). If the plasma urate is wholly filtrable at the glomerulus, it would follow that more than 90% of the filtered urate is reabsorbed in the tubules. If the plasma urate is partly or wholly non-filtrable, due to firm binding to plasma proteins or to formation of large molecular polymers, there would be correspondingly less tubular reabsorption; indeed, tubular secretion of urate might be postulated. The available data concerning the degree of filtrability of the plasma urate are conflicting. The older literature indicates complete diffusibility upon dialysis and variable results, generally 70-100% diffusibility, by ultrafiltration. Some of these records are difficult to evaluate because of sparse detail and use of now obsolete methods. More recently, Adlersberg, Grishman, and Sobotka(2) confirmed the complete diffusibility of plasma urate by compensation dialysis but, in an extensive study by ultrafiltration technics, found a mean of 16% "bound"/total uric acid (range 4-24%) in normal human subjects; higher percentages of non-filtrable urate (25-65%) were noted in 6 of 10 cases of gout, in some cases of liver disease and in a case of multiple myeloma. Bauer and Klemperer (3) stated that they could not confirm the findings of Adlersberg *et al.* but gave no details. Our own earlier experience, using the Simms-Sanders ultrafiltration apparatus in a small number of sera, indicated virtually complete filtrability of the plasma urate(4,5). On the other hand, Wolfson *et al.*(6), using a modification of the ultrafiltration apparatus devised by Lavietes(7), reported 27 and 19% nonfiltrable plasma urate, corrected for non-urate chromogen, in 2 normal human subjects studied by them.

Nonfiltrability of plasma urate of the degree reported by Adlersberg *et al.* and Wolfson *et al.* would account for only a fraction of the urate clearance deficit but the point involved is critical in respect to prevailing concepts of the renal mechanisms for urate excretion. Further study therefore seems indicated.

Methods. Two methods of ultrafiltration were employed. The first utilized the principle of the Simms-Sanders "surge" ultrafiltration apparatus(8) by which positive pressures of 200-300 mm Hg are applied to the serum samples (approximately 8 ml) contained in a specially prepared collodion tube. A rocking device containing mercury provides an alternating pressure gradient of 10 mm Hg. The resulting "surge" periodically removes the serum protein film which would otherwise occlude the filtration surface of the collodion membrane. The rate of ultrafiltration is thus greatly accelerated, 3-4 ml of ultrafiltrate being obtained in 20-30 minutes, and adsorption of urate on the protein film is minimized. Since the "surging" involves agitation of the serum, the possibility of partial denaturation of the serum proteins and liberation of loosely bound complexes was investigated by ultrafiltering serum obtained from a patient injected with 5 ml T-1824 dye. The ultrafiltrate contained no dye. The second ultrafiltration apparatus employed was that previously used by Adlersberg, Grishman, and Sobotka(2) and kindly made available to us by these investigators. This method involves filtration through "600" cellophane discs resting on metal sieve plates, using constant positive pressures of nitrogen (80 lb per sq. in.). Approximately 2 ml of ultrafiltrate are obtained in 24 hours by this method. All ultrafiltrates were tested with 10% trichloracetic

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TABLE I. Urate Content of Serum Water ($U_{s.w}$) and of Corresponding Ultrafiltrates (U_{ul}) in 10 Non-gouty Subjects (1A), 16 Gouty Subjects (1B) and 16 Gouty Subjects Receiving Benemid (1C). Ultrafiltration carried out in Simms-Sanders apparatus.

	No.	$U_{s.w.}$ (mg %)	U_{ul} (mg %)	$\frac{U_{s.w.}-U_{ul}}{U_{s.w.}} \times 100$ (%)
1A	1	6.1	6.1	0
	2	5.2	5.2	0
	3	5.8	5.8	0
	4	5.0	4.9	+2.0
	5	5.5	5.3	+3.6
	6	4.4	4.2	+4.5
	7	4.1	3.9	+4.9
	8	7.1	6.7	+5.6
	9	7.6	7.1	+6.6
	10	19.0	17.7	+7.3
				Mean = +3.5%
				S.D. = ±2.7
1B	1	11.2	11.6	—3.6
	2	8.6	8.8	—2.3
	3	8.4	8.5	—1.2
	4	10.0	9.7	+3.0
	5	11.0	10.6	+3.6
	6	7.9	7.6	+3.8
	7	8.3	8.0	+3.8
	8	10.9	10.4	+4.6
	9	10.8	10.4	+4.6
	10	8.0	7.5	+6.3
	11	11.9	11.0	+7.6
	12	8.5	7.8	+8.2
	13	11.7	10.7	+8.5
	14	9.6	8.7	+9.4
	15	12.9	11.4	+11.6
	16	10.0	8.8	+12.0
				Mean = +5.0%
				S.D. = ±5.2
1C	1	5.8	6.1	—5.2
	2	9.6	10.0	—4.2
	3	5.6	5.6	0
	4	8.9	8.9	0
	5	6.9	6.9	0
	6	7.4	7.3	+1.4
	7	5.8	5.7	+1.7
	8	6.6	6.4	+3.0
	9	6.6	6.4	+3.0
	10	8.5	8.2	+3.5
	11	8.3	8.0	+3.6
	12	6.0	5.7	+5.0
	13	10.2	9.6	+5.9
	14	6.4	5.8	+9.4
	15	6.3	5.6	+11.1
	16	5.6	4.8	+14.3
				Mean = +3.3%
				S.D. = ±5.4

acid for protein and with biuret reagent for polypeptides to exclude the possibility of membrane leakage. Urate was determined by a modification of the Buchanan, Block, and Christman method(9) incorporating the use of uricase, arsenophosphotungstic acid and urea cyanide-carbonate. The reproducibility of this method in our laboratory is in the range 5-10%. Ultrafiltrates were treated exactly the same way as serum, including addition of tungstic acid and filtration through Whatman No. 40 filter paper even though no protein was present. Uric acid concentrations in serum are expressed in terms of serum water for comparison with ultrafiltrates. The serum water content was calculated by the formula of McLean and Hastings(9a), $W_s = 99.0 - 0.75\ P_s$, a serum protein concentration of 6 g % being assumed.

Results. Table I 1A summarizes the results obtained with the Simms-Sanders ultrafiltration apparatus in 10 non-gouty subjects. The concentrations of urate in serum water are all within normal limits except for the last 3 values listed which are elevated because of azotemia associated with renal impairment. The mean difference between the concentrations of urate in serum water and those of the corresponding ultrafiltrates was +3.5%, S.D. ± 2.7%.

Table 1B summarizes the results obtained with the Simms-Sanders apparatus in 16 gouty subjects all showing distinct elevations in serum urate; 3 were in the midst of an acute attack, none were receiving drugs. There was no significant deviation in filtrability from the normal. The mean difference between the concentrations of urate in serum water and in the corresponding ultrafiltrates was +5.0%, S.D. ± 5.2%. Table 1C summarizes data on 16 additional gouty subjects, all receiving Benemid, a potent uricosuric agent(5). The increase in urinary uric acid excretion caused by such uricosuric agents as Benemid is generally believed to reflect suppression of tubular reabsorption of urate(5) but it has been suggested that they act by converting serum urate from colloidal polymer aggregates to an ultrafiltrable form(10). This hypothesis is not borne out by our data.

When the ultrafiltration apparatus employed by Adlersberg *et al.*(2) was used, however, appreciable quantities of serum urate could not be recovered in the ultrafiltrates (Table II), thus confirming their findings. The data in Table II also substantiate their

TABLE II. Urate Content of Serum Water ($U_{s.w.}$) and of Corresponding Ultrafiltrates (U_{ul}) when Ultrafiltration was Performed in Apparatus of Adlersberg *et al.* for Varying Periods of Time.

No.	$U_{s.w.}$ (mg %)	U_{ul} (mg %)	$\frac{U_{s.w.}-U_{ul}}{U_{s.w.}} \times 100$ (%)	Hr of ultrafiltration
1*	5.4	4.3	20.4	12
2†	9.3	7.9	15.6	12
3†	10.7	8.3	22.4	12
4*	4.5	3.7	16.9	18
5†	10.7	9.1	15.1	24
6†	10.5	8.5	18.5	24
7*	5.4	4.3	20.4	24
8*	4.8	2.1	55.5	30
9*	5.4	3.4	37.9	30
10*	5.1	2.1	39.5	60

* Non-gouty subject.
† Gouty subject.

TABLE III. Urate Content of Serum Water before ($U^1_{s.w.}$) and after ($U^2_{s.w.}$) Contact with Metal Sieve.

No.	$U^1_{s.w.}$ (mg %)	$U^2_{s.w.}$ (mg %)	$\frac{U^1_{s.w.}-U^2_{s.w.}}{U^1_{s.w.}} \times 100$ (%)	Hr of contact
1	5.3	4.6	13.3	6
2	5.4	4.6	15.7	12
3	7.5	6.8	9.1	14
4	7.7	6.4	17.5	18
5	7.8	6.8	12.9	18
6	7.1	5.5	22.8	24
7	5.4	4.6	15.7	24
8	7.7	6.4	22.1	24
9	6.2	5.5	15.7	24
10	7.0	1.6*	76.9	36
11	6.8	.7*	89.0	48

* Heavy bacterial growth occurred in these two sera.

notation that the discrepancy increased as the duration of ultrafiltration was prolonged. This latter observation suggested that the apparent nonfiltrability of urate might be factitious since the composition of ultrafiltrates does not ordinarily change with the time of ultrafiltration. It occurred to us that protracted contact with the supporting metal sieve could lead to degradation of uric acid, which is notoriously susceptible to alteration by a wide variety of oxidizing and reducing substances. Accordingly, samples of serum were allowed to remain in the apparatus in contact with cellophane membranes resting on the metal sieve, but without ultrafiltration by application of pressure; then the serum was withdrawn and the uric acid content redetermined. As indicated in Table III, losses in uric acid of the same order of magnitude as observed after ultrafiltration were noted; moreover, the discrepancy increased with prolongation of the experiment.† Sera kept in Erlenmeyer flasks at room temperature, without contact with the metal sieves, showed a change in urate content from +3.4% to —6.5% after 24-48 hours.

Discussion. Our results are consistent with the view that the plasma urate in man, whether present in normal quantities or in the higher concentrations encountered in gouty or uremic subjects, may be assumed to be virtually wholly filtrable at the glomerulus, and that the urate clearance deficit may properly be ascribed to some 90% reabsorption of filtered urate in the tubules. The Simms-Sanders ultrafiltration technic employed does, however, leave open the possibility that the plasma urate may be so loosely bound to plasma proteins as to be liberated in the course of ultrafiltration. As already indicated, this possibility could be excluded in the case of protein-bound T-1824 dye. Additional evidence to the contrary was obtained in the course of study of a patient with glomerulo-tubular imbalance of the Fanconi type(5). As a consequence of defective tubular transport mechanisms, this patient's kidneys were incapable of reabsorbing urate and his urate clearance was found to be equivalent to his glomerular filtration rate: $C_{urate}/C_{In} = 0.98$, as compared with a mean urate clearance ratio for normal man of 0.067. If a substantial proportion of the plasma urate were in a nonfiltrable state, this could not occur unless through the coincidence of precisely proportionate tubular secretion of urate; and the absence of any uricosuric response to Benemid in this subject makes such a coin-

† It should be pointed out, however, that there is probably more contact between the serum urate and the metal sieve in the simulated ultrafiltration experiment than when pressure is applied, since pressure would flatten out the interposed cellophane membrane. Also, the explanation offered for the discrepancy would not apply to the Lavietes apparatus in which there is no contact with a metallic support.

cidence even more improbable. In the Dalmatian hound, equivalence of the urate clearance and the glomerular filtration rate occurs regularly(11) and accounts for the excess urinary urate excretion characteristic of this species; moreover, the Dalmation hound, unlike other dogs, does not respond to uricosuric agents(12,13). In the snake and frog, Bordley and Richards(14) demonstrated equivalent concentrations of urate in plasma and capsular urine obtained by direct puncture.

The possibility that some urate may be present in the plasma in aggregates which are filtrable or readily depolymerized is not excluded by our data. Such polymers presumably would be more likely to form at the higher plasma urate concentrations found in gout or nephritis. Our data do not indicate a significant deviation from the normal diffusibility under these circumstances. In fact, gouty subjects with azotemia due to significant diminution in glomerular filtration rate often show higher C_{urate}/C_{In} ratios(15,16).

Wolfson and his collaborators(6,17) have pointed to the low urate content of cerebrospinal fluid as indicating that a substantial part of the plasma urate must be in nonfiltrable form. This does not necessarily follow since the composition of the cerebrospinal fluid does not in many respects conform to that of a plasma ultrafiltrate(18).

Summary and conclusions. 1. Sera of nongouty and gouty human subjects were subjected to ultrafiltration by 2 different procedures. The urate concentration of ultrafiltrates obtained by means of a Simms-Sanders apparatus did not differ significantly from that of the corresponding sera, expressed as serum water. Low values obtained by the second method employed were found to be factitious. 2. The available data appear to justify the assumption that the plasma urate in man is virtually wholly filtrable at the glomerulus and that some 90% of the filtered urate normally is reabsorbed in the tubules.

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Comparative Study of Three Male Genital Acid Phosphatases.* (20529)

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Kutscher and Wolbergs(1) demonstrated that the high acid phosphatase content of human seminal fluid is derived almost completely from the prostate. Since that time the biochemical properties of the human prostatic enzyme have been the subject of several investigations(2-6); in addition, monkey(7) and dog(8) prostates have been shown to possess considerable acid phosphatase activity, with moderate activity present in the rabbit prostate(9). A similar enzyme occurs in the preputial gland of the male rat(10), and high acid phosphatase activity has also been reported in the seminal vesicle of the guinea pig(9).

Because of the use of rat and guinea pig tissues in the study of steroid hormone-phosphatase relations somewhat comparable to those pertaining to the human prostate, a comparative study was undertaken of acid phosphatases from 3 male genital tract sources: human seminal plasma, rat preputial gland, and guinea pig seminal vesicle. The relations of activity to pH and to substrate concentration, as well as the effects of a series of ions and organic substances, were studied. Special attention was paid to the ability of bovine serum albumin to activate all preparations. In general, a notable degree of similarity was found.

Materials and methods. Enzyme preparations of approximately equal activity were obtained as follows: (a) prostatic acid phosphatase by dilution of human seminal plasma 1:2000 with glass-distilled water; (b) preputial acid phosphatase as the supernatant after centrifugation for 10 min. at 2000 RPM of a 0.5% aqueous homogenate of secretion-free glands from mature male Long-Evans rats; (c) vesicular acid phosphatase as a similarly prepared supernatant from the secretion-free seminal vesicles of mature male guinea pigs. Studies were made immediately after preparation of the enzyme solutions, as well as occasionally after storage at 4°C for 2-3 days. Refrigeration for this period did not result in any significant change in activity. Phosphatase activity was determined by the method of Huggins and Talalay(11), using sodium phenolphthalein phosphate as substrate and their definition of 10 units of phosphatase activity as the amount liberating one mg of phenolphthalein during one hour at 37°C. Although phenolphthalein phosphate is not a naturally occurring substrate for phosphatases, it has been demonstrated that the perfused canine prostate dephosphorylates it *in vivo*(4). Unitage curves were constructed for each of the 3 enzymes, owing to the parabolic relation between enzyme concentration and the colorimetric equivalent of the liberated phenolphthalein. 0.05 M sodium acetate-sodium cacodylate (dimethylarsenate) buffers(12) were employed in the determination of pH-activity curves from pH 4.3 to 7.4; 0.08 M sodium barbital provided the buffer at more alkaline pH's, where activity was found to be insignificant. Substrate concentration effects were studied both at pH 5.4 and at the optimum pH with 0.08 M acetate and 0.05 M phthalate buffers. Efficacy of the buffer solutions was checked during the course of incubation by means of a Beckman pH meter with portable electrodes. The effects of the following ions and organic substances were determined at pH 5.4 using a standard substrate concentration of 8.3×10^{-4} M in 0.08 M acetate buffer: Mg^{++}, Ca^{++}, Mn^{++}, Zn^{++}, F^{-}, and oxalate at a concentration of 0.017 M; molybdate, tungstate, L-tartaric acid, and bovine serum albumin (Armour) in a series of concentrations; 0.5% formaldehyde. Enzyme preparations were also tested after pretreatment with 40% ethanol for 30 min. at room temperature. Each substance

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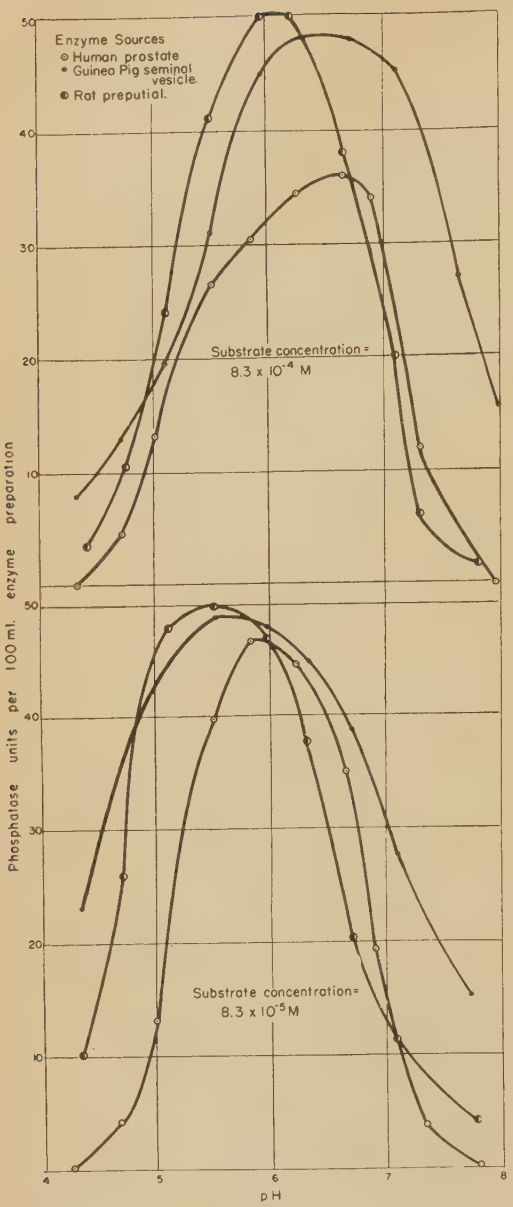

FIG. 1. pH-activity curves for human seminal, rat preputial, and guinea pig seminal vesicle acid phosphatases at 2 substrate (phenolphthalein phosphate) concentrations. Note the lower pH optima with the lower substrate concentration.

was tested several times on 2 or more preparations from each enzyme source.

Results. pH-activity curves were prepared at substrate concentrations of 8.3 x 10^{-4} M and 8.3 x 10^{-5} M. Fig. 1 shows that maximal activity occurs at a lower pH with the lower substrate concentration. In general, pH optima (5.5-6.5) are similar for the 3 enzymes. The limiting substrate concentration was around 3 x 10^{-4} M for all 3 enzymes; substrate inhibition was evident at higher concentrations. Michaelis constants were estimated from curves and were also calculated according to the method of Lineweaver and Burk(13). They were found to fall in the range of 1 x 10^{-5} to 5 x 10^{-5}; no attempt was made to determine the constants more accurately in view of the impurity of the preparations.

The metallic cations tested (Mg^{++}, Mn^{++}, Ca^{++}, Zn^{++}) inhibited the 3 enzymes to some extent; however, the results were seldom reproducible with several preparations of the same enzyme. Molybdate, tungstate, fluoride, L-tartrate, and oxalate all had appreciable inhibitory effects at the concentrations employed, except possibly at the lowest concentration of tartrate. The similar responses of the 3 enzymes to these substances are striking (Table I).

Incubation of the enzyme preparations in 40% ethanol for 30 min. at room temperature before enzyme assay resulted in total inactivation, whereas 0.5% formaldehyde had a very limited effect.

Bovine serum albumin had an activating effect over a range of concentrations. Human seminal enzyme and guinea pig vesicular enzyme were similarly activated, but male rat preputial phosphatase showed considerably less response. Thus, 0.01% albumin activates the human enzyme 235% and the guinea pig enzyme 350%, but has no effect on the rat enzyme. Only with concentrations of albumin as high as 0.1% is there an appreciable effect on preputial phosphatase (30% activation). On the other hand, 0.005% albumin still significantly activates the former (35% and 85%, respectively). A linear relation was found to exist between concentration of seminal phosphatase and the amount of activation with 0.02% albumin in the presence of excess substrate at both pH 5.4 and 6.0.

Discussion. The similarity of the pH optima (around 6.0) and of the Michaelis constants of the 3 genital acid phosphatases studied furnish some evidence for their pos-

TABLE I. Effect of Various Substances on Male Genital Tract Acid Phosphatases.*

Substance	Concentration	% inhibition of enzyme: Human seminal	Guinea pig vesicular	Male rat preputial
Sodium fluoride	1.7×10^{-2} M	95	100	100
" molybdate	10^{-4} M	93	96	95
	10^{-5} M	81	87	81
	10^{-6} M	44	58	52
" tungstate	10^{-5} M	97	95	91
	10^{-6} M	68	81	80
L-tartaric acid	10^{-2} M	88	96	96
	10^{-3} M	31	25	42
	10^{-4} M	12	13	8
Sodium oxalate	10^{-2} M	39	44	20
Formaldehyde	0.5%	17	28	16
Ethanol incubation†	40%	100	100	100

* Substrate: 8.3×10^{-4} M phenolphthalein phosphate at pH 5.4 (acetate).
† 30 min. pretreatment at room temperature.

sible identity. However, Roche(14) has emphasized that to be reliable pH optima should be obtained on purified preparations. The pH optimum of human prostatic acid phosphatase reported herein is somewhat higher than that found by some earlier workers(6, 11). However, Lundquist's detailed study (2) demonstrated an optimum as high as pH 6.3 with certain substrates (*e.g.* calcium-phosphorylcholine). In general, all 3 genital phosphatases are characterized in their impure state by pH optima higher than those ordinarily ascribed to group II phosphatases, to which they are generally assigned(14).

The lower pH optima seen when substrate concentration is reduced are of interest in view of some observations(15) on alkaline phosphatase. It was found that lowering the substrate concentration brought the optimum pH toward neutrality, a 10-fold decrease in substrate concentration diminishing the optimum by about one pH unit. However, the view(15) that the approach of the optimum to neutrality resulting from lowered substrate concentration may reflect the condition seen *in vivo,* where substrate concentrations may be low, is difficult to reconcile with our data. With the acid phosphatases, lowered substrate concentration results in a pH optimum further removed from neutrality.

The variable results obtained with the metallic ions may possibly be due to their occurrence in the enzyme preparations themselves; appreciable calcium is found in human seminal plasma(16). An inhibition of the prostatic enzyme by Mg^{++} was repeatedly found, despite the presumed lack of effect of this ion on group II phosphatases(14).

The effects of certain anions and organic substances provide data of comparative value (Table I). The well established inhibitors of group II acid phosphatases: fluoride(14), molybdate and tungstate(17), and oxalate (5,18), had similar effects on all preparations. The human prostatic enzyme can be distinguished from isodynamic serum acid phosphatases by its sensitivity to L-tartrate (3,19-21) and to ethanol(22-24) and its resistance to formaldehyde(20,24,25). These 3 substances have similar influences upon the guinea pig and rat genital enzymes, despite their origin from sex accessories which are not homologous to the human prostate.

Acid phosphatase from the male rat preputial gland is activated by 0.1% albumin to about the same extent as β-glucuronidase(26) from the female rat preputial gland (31% as compared with 39%). In attempting to account for the considerably greater activation of human seminal and guinea pig vesicular acid phosphatases, it should be noted that the rat preputial homogenate is prepared from a very different type of tissue, consisting of holocrine acini with a high lipid content(27).

The marked increase in prostatic acid phosphatase activity in the serum generally diagnostic of metastasizing human prostatic carcinoma(28) may be related in part to the

activation of the enzyme by serum albumin. In addition, a thermostable, low molecular weight organic activator of prostatic acid phosphatase has been found in serum(29). Although alkaline hyperphosphatasemias generally reflect an actual enrichment by the enzyme, rather than an activation(30), the latter possibility in regard to prostatic acid phosphatase in human serum seems worthy of consideration.

Summary and conclusions. Three male genital tract acid phosphatases, from human seminal plasma, rat preputial gland and guinea pig seminal vesicle, show a high degree of similarity in their pH-activity curves, Michaelis constants, and reactions to certain anions and organic substances. As a group these enzymes are fluoride-, tungstate-, molybdate-, oxalate-, L-tartrate-, and ethanol-sensitive and formaldehyde-resistant. All are activated by serum albumin, the preputial enzyme less than the other two.

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Metabolic Interrelationship between Folic Acid, Vitamin B_{12}, and the Citrovorum Factor.* (20530)

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Present evidence indicates the presence in avian liver of 2 enzyme systems which are responsible for an increase in microbiologically determinable citrovorum factor (CF). A report by Hill and Scott(1) suggests that ascorbic acid increases the CF content of chick liver homogenate by activating a CF conjugase present in chick liver. Nichol(2) reported that formation of CF from pteroylglutamic acid (PGA) in chick liver homogenate at an optimal pH of 6.2 proceeded well under anaerobic conditions. Recently, Broquist *et al.*(3) reported 39% to 54% conversion of added PGA to CF by resting cells of a strain of *S. faecalis* resistant to 4-amino-10 methyl pteroylglutamic acid(4). Such high enzymatic formation of CF was obtained only when the resting cells were incubated with PGA and a supply of "formate" in presence of a reducing environment.

The metabolism of "formate" is influenced by dietary PGA according to reports by several groups(5-8). Reports have appeared to indicate a participation of vit. B_{12} in the conversion of PGA to CF(9-11) but the evidence does not seem conclusive. It is the purpose of this report to determine the effect of supplementing the chick diet with varying levels of vit. B_{12} and PGA on the *in vitro* synthesis of CF from added PGA by the liver homogenates.

Experimental. Straight run (New Hampshire males X Single Comb White Leghorn female) crossbred chicks were used for all the studies. Birds were housed in electrically heated batteries with raised screen floors. The chicks were wingbanded, weighed and randomized into 12 groups of 12 birds each at one day of age. Weights were recorded at weekly intervals. The basal diet used in the present studies was the same as reported by Couch *et al.*(12) with the exception that the diet contained 69.5% Cerelose, 22.5% soybean protein (Drackett 220), 2.5% soybean oil, 6 mg/kg α-tocopherol, 10,000 I.U. vit. A/kg, and 2,000 I.C.U. vit. D_3/kg. PGA and vit. B_{12} were not added to the basal diet since the latter was supplemented with 3 different levels of PGA (2, 100, 400 mg/kg) and 2 levels of vit. B_{12} (30, 500 γ/kg).

At the end of the 4-week period, 3 chicks from each group were sacrificed, the livers were removed and immediately chilled with ice for 2 minutes; 20% homogenates were then prepared in 0.08 M sodium potassium phosphate buffer pH 6.3, using the Potter-Elvehjem glass homogenizer. Equal amounts of the 3 homogenates were mixed and representative homogenates thus obtained from the various groups of birds were immediately tested for PGA to CF conversion studies as follows: 5 ml aliquots of the various homogenates were pipetted in duplicates into 50 ml Erlenmeyer flasks containing 5 ml of the above mentioned phosphate buffer and also aliquots of solutions containing substances to be described. The final volume in each flask was made to 11 ml with water and the solutions were layered with toluene. Then the flasks were tightly rubber-stoppered and nitrogen was introduced by the use of hypodermic needles thrust through the rubber stoppers (13). After flushing with nitrogen, the needles were carefully withdrawn and the flasks were incubated on a reciprocating shaker at 37°C for 2 hours. The rubber stoppers were removed and replaced by the usual

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TABLE I. Conversion of PGA to CF by Liver Homogenates from Chicks Fed a Low Vit. B_{12}-PGA Diet Supplemented with Varying Levels of Vit. B_{12} and PGA.

Group No.	Supplements to basal diet: PGA, mg/kg	Supplements to basal diet: Vit. B_{12}, γ/kg	Vit. B_{12}, mγ/g liver	γ PGA added/flask*: 0	50	100	250
				γ CF/g liver homogenate			
1	0	0	18	1.3	13.6	25.8	45.5
2	0	30	129	1.4	17.1	30.0	55.4
3	0	500	364	3.2	27.5	43.4	64.9
4	2	0	12	3.6	17.3	25.2	61.0
5	2	30	165	3.7	23.4	45.8	55.0
6	2	500	340	12.8	36.7	66.1	75.6
7	100	0	46	2.7	20.0	30.2	62.5
8	100	30	286	9.6	33.4	51.0	71.3
9	100	500	611	8.5	37.4	72.5	121.0
10	400	0	18	4.2	25.8	39.5	67.4
11	400	30	256	10.7	43.6	71.8	108.8
12	400	500	406	12.6	31.9	44.6	54.5

* Flasks components: 5 ml of 20% liver homogenate in .08 M sodium-potassium phosphate buffer pH 6.3 along with 5 ml of the same buffer and aliquots of PGA solution, made to a total volume of 11 ml with H_2O. Flasks were incubated for 2 hr under N_2.

cotton plugs and all the samples were autoclaved for 5 minutes at 120°C. After cooling, the samples were homogenized in a Waring Blendor, neutralized, made up to volume, filtered, and the filtrate was assayed. The CF content was determined using the single strength basal medium VI of Steele *et al.*(14) with acid hydrolyzed casein to supply part of the amino acids. *Leuconostoc citrovorum* ATCC 8081† was used as the test organism and Leucovorin (Lederle) was employed as the standard. Since the latter has been reported to be half as active as the CF isolated from horse liver(15), the microbiological assay values were divided by 2 in order to express the results in terms of the naturally occurring CF. The cultures were incubated for 72 hours at 37°C and growth was determined by titrimetric procedure.

Liver samples of 6 chicks from each group were pooled and the vit. B_{12} determinations were run in triplicates. The method of Yacowitz *et al.*(16) modified by addition of 50 γ KCN per g of liver homogenate was used for liberating vit. B_{12}(17). Determinations of vit. B_{12} were made on the treated liver samples using the titrimetric method of Skeggs *et al.* (18) with *Lactobacillus leichmannii* 4797 as the test organism and the crystalline vit. B_{12} as the standard.

Results. It is apparent from the results of Table I that supplementing of chick diet either with increasing amounts of vit. B_{12} or PGA increased the conversion of added PGA to CF by the liver homogenate. This is largely true of results from chicks of Groups 1 to 11 while on the other hand liver homogenates from chicks receiving highest levels of vit. B_{12} and PGA (Group 12) gave a lowered PGA to CF conversion. Also homogenates of liver from chicks receiving 400 mg PGA/kg of diet (Group 10) gave an increase in PGA to CF conversion which was comparable to supplementation of the diet with 500 γ vit. B_{12}/kg (Group 3). This would suggest that the 2 vitamins may influence the converting mechanism by separate pathways.

It was of interest to determine if the CF synthesized by chick liver homogenate from added PGA was identical with the synthetic CF (Leucovorin). This was accomplished by analyzing the filtrate prepared for CF assays, using bioautographic procedures described by Doctor and Couch(19). Paper strips were developed using wet symmetrical collidine and also using a solvent system composed of 50%

† A recent report (Felton, E. A., and Niven, C. F., *J. Bact.*, 1953, v65, 482) concerning a taxonomic study on the culture *Leuconostoc citrovorum* ATCC 8081 suggests that the latter organism is a typical strain of *Pediococcus cerevisiae* as described earlier by Pederson (*Bact. Rev.*, 1949, v13, 225).

TABLE II. Influence of Adding Vit. B_{12} and Formate on Conversion of PGA to CF by Liver Homogenates from Chicks Fed a Low Vit. B_{12}-PGA Diet Supplemented with Varying Levels of Vit. B_{12} and PGA.

Group No.	Supplements to basal diet		Substances added/flask*			
	PGA, mg/kg	Vit. B_{12}, γ/kg	None	PGA, 50 γ	Formate, 10 mg + vit. B_{12}, 10 γ	Formate, 10 mg + vit. B_{12}, 10 γ + PGA, 50 γ
			γ CF/g liver homogenate			
1	0	0	1.3	13.6	1.1	17.1
2	0	30	1.4	17.1	1.3	20.3
3	0	500	3.2	27.5	3.8	30.3
4	2	0	3.6	17.3	3.4	23.6
7	100	0	2.7	20.0	2.9	17.8
10	400	0	4.2	25.8	4.5	28.0

* Flask components: 5 ml of 20% liver homogenate in .08 M sodium potassium phosphate buffer pH 6.3 along with 5 ml of the same buffer and aliquots of solutions containing substances described to make a total volume of 11 ml. Flasks were incubated for 2 hr under N_2.

ethanol, 15% *n*-butanol, 10% ammonia, and 25% water(19). Results of both these studies indicated that the compound synthesized from PGA by chick liver homogenate moved on paper strips at the same rate as did synthetic CF (Leucovorin).

Supplementing the chick diet with increasing amounts of vit. B_{12} increased by many fold the vitamin content in the liver (Table I). Supplementing with 100 mg PGA/kg also resulted in an increased storage of vit. B_{12} in the liver. Supplementation of the basal diet with 400 mg PGA/kg produced a slight decrease in the vit. B_{12} content of the livers as compared to values obtained when 100 mg PGA/kg were fed. The soybean protein used in the basal ration supplied 50 γ PGA/kg of diet and 15 γ CF/kg. Average weights of the birds at 4 weeks of age ranged from 293-355 g. No apparent differences were obtained which could be correlated with dietary changes.

In order to investigate whether feeding a diet low in vit. B_{12} resulted in a low storage of "formate" in the liver and to determine if vit. B_{12} is directly concerned in some way with the converting mechanism, studies were conducted to determine the influence of *in vitro* addition of vit. B_{12} and "formate" on the PGA to CF conversion. Results of this study presented in Table II show only a slight stimulation of PGA to CF conversion by *in vitro* addition of 10 γ vit. B_{12} together with 10 mg sodium formate. The data may be interpreted to suggest that vit. B_{12} may have to be converted into a type of coenzyme system concerned with the synthesis of CF from added PGA but the failure of added formate in giving an appreciable increase in the conversion is even more intriguing(3,20).

Discussion. Investigations by Drysdale *et al.*(6) where PGA deficiency decreased the incorporation of formate into C-2 and C-8 of the liver adenine and guanine and that of Skipper *et al.*(7), in which the incorporation of formate into total nucleoprotein of mice is decreased by antagonist-induced PGA deficiency suggest a probable CF-catalyzed utilization of formate in the synthesis of purines(21). Recently, Buchanan and Schulman(20) have reported a more direct influence of CF in a preferential incorporation of radioactive formate into C-2 of inosinic acid. It is apparent that under the presently described experimental conditions an increased conversion of PGA to CF is obtained in liver homogenates from chicks receiving increasing levels of PGA or vit. B_{12}. In order to ascertain if this increase in conversion was largely due to a general increase in metabolic activity of the liver, studies on the endogenous oxygen uptake were conducted. It was observed that supplementation with vit. B_{12} or PGA did not appreciably affect the endogenous oxygen uptake.

Since vit. B_{12} is not effective in reversing the effects of certain PGA analogues which are known to block the transformation of PGA to CF, it is conceivable that dietary vit. B_{12} and PGA may in some way prevent

the inactivation of CF synthesized by the liver homogenate and thereby cause an apparent increase in CF production. The evidence does not, however, rule out the possibility that a specific effect of vit. B_{12} and PGA is involved.

Summary. Supplementing a low vit. B_{12}-PGA chick diet with either increasing levels of vit. B_{12} (30 γ and 500 γ/kg) or increasing levels of PGA (2, 100, and 400 mg/kg) resulted in an increased capacity by the liver homogenates to convert added PGA to CF. The results further suggest that the 2 vitamins may influence the converting enzyme system through different mechanisms. *In vitro* addition of formate and vit. B_{12} to the liver homogenate gave only a slight stimulation of PGA to CF conversion by the liver homogenate.

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Effect of Aureomycin upon Growth and Maturation of *Lebistes reticulatus.* (20531)

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Recent work in nutrition has indicated that addition of aureomycin to the diet increased the rate of growth of newborn or immature animals(1-7). Jukes *et al.* produced acceleration of growth in pigs using aureomycin supplemental feeding(1). These results have been confirmed and extended to other animals as the chick(2-7). Growth-enhancing effect of aureomycin in premature children has also been described(8). Recently it has also been shown that aureomycin supplemental feeding enhanced the growth of rats made diabetic with alloxan(9).

The above work demonstrating the anabolic effect of the broad spectrum antibiotic in mammals and birds and in experimentally induced diabetes made it of interest to study this propensity of the drug in lower vertebrates. To this end the experimental report herein is concerned with the observations on

TABLE I. Aureomycin upon Growth and Maturation of *Lebistes reticulatus.*

	Length (mm)	Width (mm)	Wt (mg)	Sex	Gonad differentiation
Controls					
	15	4	61	♂	Immature—no mature sperm
	19	5	127	♀	Mature ovary with large yolk egg
	17	4	109	♀	*idem*
	18	5	133	♀	*idem*
	15	4	69	♂	Immature—beginning of mature sperm formation
	15	4	77	♂	——
	20	5.5	178	♀	——
Avg	17	4.5	107.7		
Exp.					
	14	3	45	♂	Mature with mature sperm
	13	3	36	♂	Immature—no mature sperm
	13	3	36	♀	" —no large yolk eggs
	11	3	21	♂	Mature with mature sperm
	13.5	3	41	♀	Immature—no large yolk eggs
	10.5	3	32	♀	*idem*
Avg	12.5	3	35.2		

the effect of aureomycin supplemental feeding upon the growth and development of the teleost fish, *Lebistes reticulatus,* more commonly known as the guppy.

Method. Newborn guppies were divided into 2 groups, both of which were raised under identical conditions with the exception that the experimental fish received supplemental feeding of aureomycin hydrochloride* equal to 20% of the weight of the added food. Both groups received equal aliquots of a commercially prepared fish food. After a period of 6 months the experimental and control fish were sacrificed, measured and weighed to the nearest milligram. The fish were then fixed in Bouin's solution, embedded in paraffin, and sectioned to determine the effects, if any, of the aureomycin feeding on the development and differentiation of the gonads.

Results. Contrary to previously reported work on mammals and birds, we found that aureomycin supplemental feeding in the described doses inhibited the growth of guppies. Table I summarizes the individual measurements, weights and sexual differentiation of the control and experimental groups.

The average size and weight of the 7 control animals and 6 aureomycin-fed animals are as follows: Length from tip of snout to base of caudal fin was 17 mm for the control group and 12.5 mm for the experimental group. The width at its widest point was 4.5 mm for the control group and 3 mm for the experimental group. The weight of the control group was 107.7 mg, while that of the experimental group was only 35 mg. This represents almost a 70% inhibition of growth as measured by the weight of the animals. The failure of the experimental group to approach the size of the control animals as evidenced by the length and width indicates that both a growth and anabolic inhibition were attained. Microscopic examination showed no consistent significant effect on gonadal growth or differentiation. It is of interest to note that despite the retardation of somatic development, examination of the gonads showed no evidence of inhibition in these animals since a comparable differentiation was noted in both the experimental and control fish.

A point of interest was the development of a mold growth determined to be *Monilia albicans* in the tank containing the aureomycin.

Discussion. These data on the suppression of growth of fish with aureomycin supplemental feeding are contrary to the published effects of similar feedings on mammals and birds. They are, however, in accord with the work of Lepine *et al.,* who inhibited the growth of tissue culture by the addition of

* The aureomycin hydrochloride used in this study was kindly furnished by Lederle Laboratories Division of American Cyanamid Co., N. Y.

aureomycin to the media(10). The observations in guppies are somewhat in agreement with the more recent work on the catabolic effects of aureomycin in man and dogs(11). We cannot at this point explain why aureomycin feeding stimulates growth in mammals and birds and inhibits growth in fish. It is yet to be determined whether these results are due to specific differences or based upon the possible catabolic propensities of large doses of aureomycin as previously described (11). An overgrowth of a mold identified as *Monilia albicans* was noted in the water of the aquarium in which aureomycin had been added. The increased growth of Monilia noted in the tank after the addition of aureomycin is in accord with some clinical observations on the apparent increase in moniliasis after the use of broad spectrum antibiotics. One must consider the possibility that this fungal growth might be a factor in inhibiting somatic growth in the fish.

Summary. Supplemental feeding of aureomycin to guppies caused a marked inhibition of somatic development and growth. There was, however, no gonadal inhibition. The possible mechanism of this action of aureomycin was discussed.

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Comparative Effects of Intramuscular Injections of ACTH, Cortisone, and Saline on Serum Glycoprotein Levels.* (20532)

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(Introduced by Charles M. Carpenter)

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Increases in the protein-bound carbohydrates of serum have been observed in such diverse conditions as human(1) and experimental(2) tuberculosis, human(3) and experimental(4) carcinoma, human(5) and experimental(6) thermal injury, late pregnancy and parturition(7), rheumatic fever and osteomyelitis(8), X-irradiation(9), immunization(10), experimental scurvy(11), and from the administration of parathyroid extract(12). The reports suggest that the elevated serum glycoprotein levels may represent a response to non-specific stress(13).

Although the mechanism is still speculative, it is now generally accepted that the pituitary-adrenal axis is concerned in some fundamental way with the ability of the organism to respond to stress. A wide variety of unrelated stimuli such as heat, cold, tissue injury, hemorrhage, infections, the injection of bacterial toxins, toxic chemicals, foreign proteins, epinephrine, thyroxine, and insulin have been found to augment the rate of secretion of

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TABLE I. Summary of Chemical Analyses.

Group	A	B	C	D
No. of animals	27	22	20	24
Treatment	None	10.0 mg ACTH/day	10.0 mg cortisone/day	0.5 ml saline/day
Avg wt before treatment		653	595	573
Avg wt after treatment		620	622	597
Total serum* polysaccharide (mg %)	111 ±2.5	127 ±4.3	119 ±3.8	131 ±6.0
Mucoprotein* polysaccharide (mg %)	29 ±1.1	31 ±1.1	27 ±1.7	38 ±2.5
Total serum* protein (g %)	5.4± .08	5.2± .09	5.4± .10	4.9± .09
Total serum poly.*/Total serum protein × 100 (%)	2.1± .05	2.4± .07	2.2± .08	2.7± .11

* Including stand. error of the mean calculated from the relation $E_m = [\Sigma d^2/(N(N-1))]^{1/2}$ where "d" is the deviation from the mean and N the No. of observations.

TABLE II. Statistical Comparison of Results.

Determination	Groups compared	Difference between means	D.F.	t	P
Total serum polysaccharide	Normal (A) vs. ACTH-treated (B)	16 mg %	47	3.312	<.01
	" " " cortisone-treated (C)	8	45	1.824	.1
	" " " saline-treated (D)	20	49	3.180	<.01
	Saline-treated (D) vs. ACTH-treated (B)	4	44	0.528	.6
	" " " " cortisone-treated (C)	12	42	1.608	.2
Mucoprotein polysaccharide	Normal (A) vs. ACTH-treated (B)	2 mg %	47	1.284	.3
	" " " cortisone-treated (C)	2	45	1.044	.4
	" " " saline-treated (D)	9	49	3.672	.001
	Saline-treated (D) vs. ACTH-treated (B)	7	44	2.653	<.02
	" " " " cortisone-treated (C)	11	42	3.696	.001
Total serum protein	Normal (A) vs. ACTH-treated (B)	.2 g %	47	1.685	.1
	" " " cortisone-treated (C)	.0	45	0.000	—
	" " " saline-treated (D)	.5	49	4.123	.001
	Saline-treated (D) vs. ACTH-treated (B)	.3	44	2.385	<.05
	" " " " cortisone-treated (C)	.5	42	3.616	.001
Total serum polysaccharide ÷ total serum protein × 100	Normal (A) vs. ACTH-treated (B)	.3 %	47	3.561	.001
	" " " cortisone-treated (C)	.1	45	1.144	.3
	" " " saline-treated (D)	.6	49	5.136	.001
	Saline-treated (D) vs. ACTH-treated (B)	.3	44	2.175	<.05
	" " " " cortisone-treated (C)	.5	42	3.470	<.01

pituitary adrenotrophic hormone(14).

If the increased secretions of ACTH and adrenal cortical hormones following stress are responsible for the elevation of serum glycoprotein levels, then the administration of the hormones to normal animals might be expected to cause an increase in the concentrations of the circulating conjugated proteins. The present investigation was undertaken to study the effects of exogenous ACTH and cortisone on the total serum glycoprotein, serum mucoprotein, and total serum protein levels of normal guinea pigs. Sterile 0.85% sodium chloride was administered to control animals in order to evaluate the stress effects of the injection procedure.

Experimental. Male and female guinea pigs were maintained *ad libitum* on a diet of rabbit pellets plus greens, and tap water. Injections of 5 mg ACTH,† 5 mg cortisone,‡ and 0.25 ml of 0.85% saline were made intramuscularly, at 12-hour intervals, for 11 days. After the final injection, the animals were bled by cardiac puncture, the sera separated and stored in the frozen state until the chemical analyses could be conducted.

† The ACTH (Actrope) employed in the investigation was obtained through the courtesy of Mr. S. S. Kingman and Mr. Richard C. Bruner, United Laboratories, Ltd., Pasadena, Calif. One mg of Actrope was equivalent to one U.S.P. unit.

‡ Cortone acetate, Merck and Co., Rahway, N. J.

Total serum polysaccharide, mucoprotein polysaccharide and total serum protein were determined as previously described(2). The total serum polysaccharide and mucoprotein polysaccharide values were employed as indices of the levels of the total serum glycoprotein and serum mucoprotein, respectively. For comparative purposes, the data obtained from the serum analyses of normal, untreated animals and which have been previously reported(2) are included. The group means for all determinations were compared by the "t" test of Fisher(15).

Results. The summary of the results of the chemical analyses and weight determinations is presented in Table I. A statistical comparison is shown in Table II. *Total serum polysaccharide.* Statistically significant increases were observed when the means of the groups which received ACTH (B) and saline (D) were compared with the mean of the untreated animals of Group A. The elevation that occurred in the cortisone-treated group (C) was not significant. No significant differences were found when the means of the groups administered hormones (B, C) were compared with the average of the saline-treated guinea pigs of Group D. The individual values within the group which received saline (D) exhibited a greater degree of variability as indicated by the magnitude of the standard error of the mean. *Mucoprotein polysaccharide.* The serum mucoprotein values remained within the normal range for the groups administered ACTH (B) and cortisone (C). The guinea pigs of Group D, which received saline, exhibited a significant increase in mucoprotein polysaccharide when compared with the untreated animals of Group A. The serum mucoprotein content of the saline-treated group (D) also differed significantly from the means of the groups (B, C) which received injections of hormones. *Total serum protein.* A statistically significant decrease occurred in the total serum protein content of Group D, which received saline (Table II). The mean of the cortisone-treated group (C) was identical with the average value of the untreated guinea pigs of Group A. Group B, which was injected with ACTH, exhibited a small, but non-significant decrease in total serum protein. *Total polysaccharide as % of protein.* Highly significant increases were observed in the total serum polysaccharide levels with respect to the total serum protein in the ACTH (B) and saline-treated (D) groups. In the case of Group D, the increased percentage was due in part to the marked decrease in the amount of circulating protein. *Weight.* The weight responses of the hormone-treated groups (B, C) were similar to those previously observed in uninfected guinea pigs which received the dosages of ACTH and cortisone employed in the present study(16). The effect of saline administration approximated that of cortisone, causing a small retardation in the normal rate of growth.

Discussion. Qualitative and quantitative differences have been observed with respect to total serum glycoprotein, serum mucoprotein, and total serum protein following the administration of ACTH, cortisone, and saline to normal guinea pigs. The total serum glycoproteins were elevated in the 3 groups which received injections, the increases being of statistical significance in the ACTH- and saline-treated groups. Serum mucoprotein values were within the normal range for the groups which received hormones, but were significantly elevated for the group administered saline. A significant hypoproteinemia was observed in the guinea pigs injected with saline. The decrease in the total serum protein did not occur in the groups administered hormones.

Since the concentrations of total serum glycoprotein and serum mucoprotein of the hormone-treated groups were less than the corresponding serum levels in the animals which received saline, the conclusion may be drawn that increased secretions of ACTH and cortisone are not directly responsible for the elevation of serum glycoprotein levels observed in many types of stress.

The pattern of alterations in the serum components of the saline-treated guinea pigs, presumably due to the trauma and emotional stimuli of the injection procedure, was remarkably similar to the serum changes reported for patients with neoplasms(3) and tumor-bearing rats(4). The deviations from

the normal serum values observed in the saline-treated group were partially prevented by the administration of ACTH or cortisone. A homeostatic effect of the hormones was suggested since the concentrations of serum mucoprotein and total serum protein in the groups which received ACTH or cortisone, did not differ significantly from the corresponding values of the untreated control group.

The studies reported above support in part the concept, emphasized by Roberts(17), that the primary role of the adrenal cortical secretions in protein metabolism is stimulation of the mobilization of tissue protein. Roberts (17) has demonstrated that the depression of total serum protein following partial hepatectomy in the rat may be prevented by treatment with ACTH or ACE. In the present investigation, the hypoproteinemia which followed the intramuscular injection of saline did not occur in the guinea pigs which received ACTH or cortisone. The data obtained from the total serum glycoprotein and serum mucoprotein determinations are not in complete agreement with this interpretation. If the labile protein-bound carbohydrates of tissues were released under hormonal influence as postulated above, greater concentrations of the conjugated proteins should be present in the sera of the hormone-treated animals than in the circulation of the guinea pigs which received saline. The observation that the serum glycoprotein and serum mucoprotein levels of the saline-treated group were higher, suggests that more than one physiologic mechanism may be involved in the metabolism of the glycoproteins and the carbohydrate-free proteins of serum.

Summary. The effects of intramuscular injections of ACTH, cortisone, and 0.85% sodium chloride on total serum glycoprotein, serum mucoprotein, and total serum protein levels of normal guinea pigs have been investigated. Statistically significant increases in total serum glycoprotein were observed in the ACTH- and saline-treated groups. Serum mucoprotein values were significantly elevated and the total serum protein values significantly decreased in the group which received saline. The conclusion was drawn that increased secretions of ACTH and cortisone are not directly responsible for the elevated total serum glycoprotein and serum mucoprotein levels following stress.

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Bacterial Oxidation of Salicylate and Related Antirheumatic Phenolic Acids.* (20533)

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In the course of tests of various antibiotic and bacteriostatic agents for their toxicity to Daphnia(1), it was shown that when the animals were placed in watery solutions of the test materials and were otherwise starved they generally survived at best not more than 2 weeks. A notable exception was observed in experiments with salicylates. Daphnia regularly lived for several weeks, and often multiplied, in M/1000 solutions of salicylate. Inasmuch as this substance acts for the most part as a protoplasmic poison, the mechanism of its beneficial effect in this instance was not immediately apparent but has since been traced to the presence of bacteria which served as food for Daphnia and proved to be capable of growth in media containing as much as M/10 salicylate as the sole carbon source. It was found that entirely similar microorganisms could be recovered readily at any time from dilute solutions of salicylate and ammonia allowed to stand open at the window for a day or two.

Interest in the systematic study of these bacteria stems in large part from the possibility that their biochemical activities might provide information about the metabolic fate of the phenolic acids used clinically as antirheumatic drugs, *viz.*: salicylate, gentisate, and 2:3-dihydroxybenzoate(2). This report concerns an attempt to apply to the problem some of the principles and methods developed in recent years by Stanier(3,4), Happold(5), Evans(6), and others(7-9), for analysis of bacterial fission of other aromatic compounds.

Materials and methods. The bacteria used, designated arbitrarily as strains 929A and 616, are Gram-negative aerobic bacilli capable of vigorous growth at 20°C on simple media and in a wide variety of substrates, including citrate. The media contained M/15 phosphate buffer pH 6.5, 0.1% NaCl, 0.1% ammonium sulfate, 0.05% $MgSO_4 \cdot 7H_2O$, and Fe SO_4 10^{-8} w/v. After this solution was autoclaved, the aromatic substrates, sterilized by filtration, were added to a final concentration of 0.1%. Strain 929A grew well on salicylate and gentisate and was used for the studies of the oxidation of these compounds. It did not, however, grow on 2:3-dihydroxybenzoic acid.‡ Strain 616 was recovered from a solution of 2:3-dihydroxybenzoic acid allowed to stand open for several days. When isolated in pure culture it was found to grow well only if pantothenate was added in trace amounts. For the cultivation of strain 616, therefore, pantothenate was added to the media in a concentration of 10^{-6} w/v.

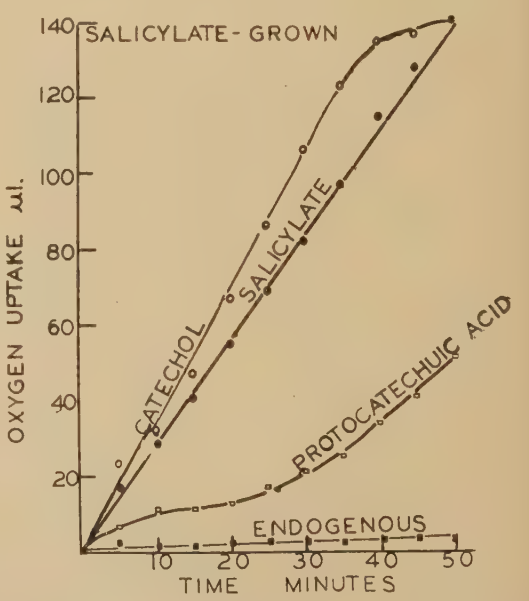

FIG. 1. Oxidation of salicylate, catechol, and protocatechuic acid by strain 929A grown on salicylate.

* This study was supported by a grant from the U. S. Public Health Service (G-3206).

† American Cancer Society Fellow.

‡ The Smith, Kline and French Co. kindly supplied the 2:3-dihydroxybenzoate.

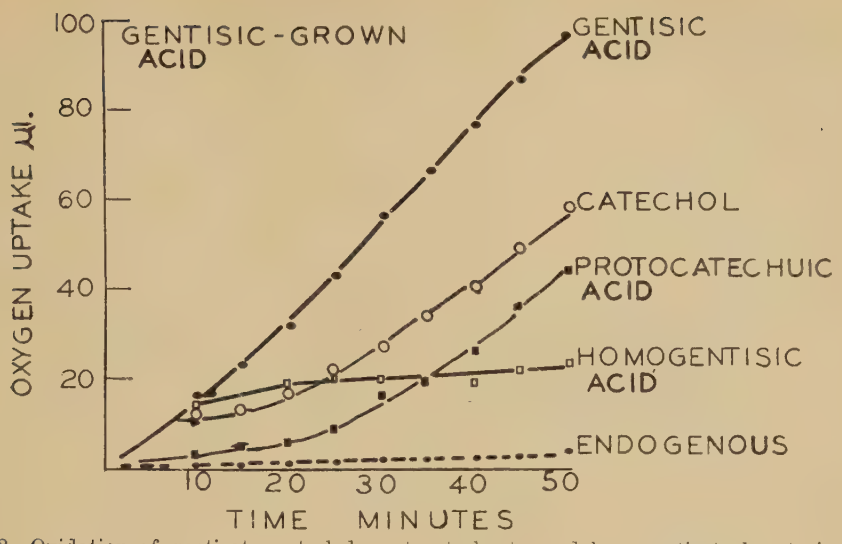

FIG. 2. Oxidation of gentisate, catechol, protocatechuate, and homogentisate by strain 929A grown on gentisate.

The manometric measurements were carried out according to the methods of Stanier(3), using washed bacteria harvested after 24-48 hours' growth on agar plates at 20°C. The bacteria were suspended in M/15 phosphate buffer pH 6.0 to a standard optical density. The Warburg vessels contained in the main compartment 2.0 ml of bacterial suspension, in the side arm 0.2 ml of .01 M substrate. Oxygen uptake was measured in air at room temperature 23-25°C for about 90 minutes.

Qualitative chemical tests were made on filtered aliquots of liquid media. The reaction for β-diketones was carried out with the reagents of Witter, Snyder, and Stotz(10), while the amounts of gentisate were estimated from the color developed upon addition of Folin's uric acid reagent(11).

Results. The pattern of simultaneous adaptation for organisms grown on salicylate is shown in Fig. 1. The rate of oxidation of salicylate is equalled and even a trifle exceeded, by the rate of oxidation of catechol. At the same time, protocatechuate is oxidized very slowly.

It is important at this point to note the essentials of Stanier's(3) postulates: 1) If the dissimilation of A proceeds through a series of intermediates, B, C, D, etc., and if the individual steps in this chain of reactions are under adaptive enzyme control, then growth on A will produce cells simultaneously adapted to A, B, C, D, etc. 2) If growth on A fails to adapt the cells to X then X cannot be a member of the reaction chain. 3) Growth on C adapts to D but not necessarily to A and B.

Applying these ideas here (Fig. 1), it appears that organisms grown on salicylate are simultaneously adapted to catechol, which thus becomes a probable intermediate in the reaction chain. On the other hand there is no adaptation to protocatechuate. Other substrates, not shown in Fig. 1, to which salicylate-trained bacteria demonstrated no adaptation included gentisic acid, homogentisic acid, gallic acid, and pyrogallol. It appears, therefore, that the most probable pathway of salicylate oxidation is in this instance through catechol. Indeed, it has been suggested(4b) that bacterial oxidation of salicylate might involve its transformation to catechol, though an experimental demonstration of this has apparently been lacking.

Bacilli grown on gentisate showed no adaptation to catechol, protocatechuate, or homogentisic acid (Fig. 2). Nor did they oxidize hydroquinone. Entirely similar negative findings were obtained when strain 616 was grown on 2:3-dihydroxybenzoic acid and tested for its adaptation to possible intermediates (Fig. 3). It therefore appeared that

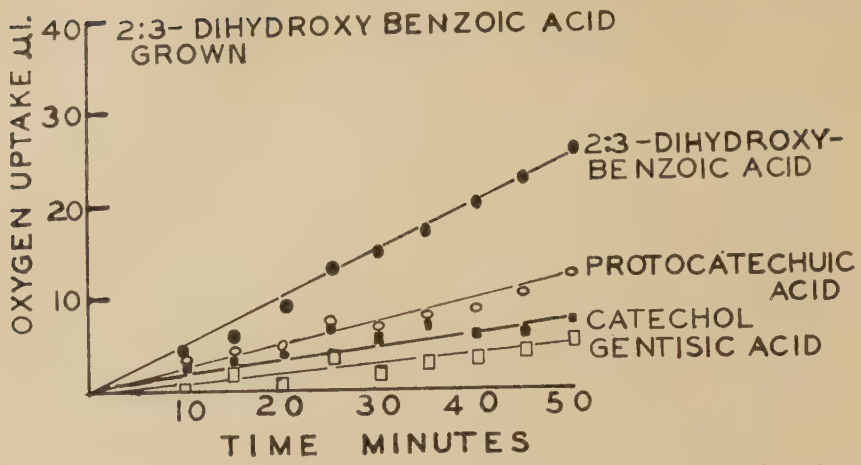

FIG. 3. Oxidation of 2:3-dihydroxybenzoate, protocatechuate, catechol, and gentisate by strain 616 grown on 2:3-dihydroxybenzoate.

the oxidation of gentisate and of 2:3-dihydroxybenzoic acid did not follow any of the pathways yet described for aromatic compounds. It was then hypothesized that the oxidation here might involve simultaneous rupture of the aromatic ring as in the case of homogentisic acid(12-14) with production from gentisate of a straight-chain β-diketodicarboxylic acid, $C_7H_6O_6$.

Accordingly, qualitative chemical tests were performed to see if any support for this idea could be found. Liquid media containing gentisate as substrate were inoculated with strain 929A and examined at intervals thereafter. It could be shown that as gentisate disappeared there developed some substance or substances forming an orange precipitate on addition of 2:4 dinitrophenylhydrazine in acid solution. Most interesting, however, was the appearance of a reaction with o-phenylenediamine. This was maximal after some 96 hours of bacterial growth, and consisted of 2 distinct visible phases. On addition of filtered medium to an equal part of the o-phenylenediamine reagent(10), a purple precipitate settled out slowly. The supernatant separated from this had the reddish tint which is characteristic of the reaction of β-diketones(10). Efforts to isolate the substances giving these reactions are incomplete; it has been difficult to separate them from gentisate because of its similar solubility properties. It would clearly be premature to suggest more than that the findings are consistent with the presence of ketones, including β-diketones, and that these could well be products of the oxidation of gentisate. The possibility was considered that they are not such, but are instead the result of bacterial synthesis. However, media containing other substrates, *e.g.*, salicylate, were examined in the same fashion and showed no comparable reaction with o-phenylenediamine.

Preliminary tests of media containing 2:3-dihydroxybenzoic acid and inoculated with strain 616 give findings entirely similar to those obtained with gentisate, and suggest again the formation of β-diketones.

Discussion. Insofar as the specific problem of the metabolic fate of antirheumatic substances is concerned, the demonstration that salicylate may be oxidized to catechol probably has little or no significance. For, in the first place, it is not known that this reaction can occur in man, although it is said that catechol appears in the urine of horses(15). Secondly, gentisate, an effective antirheumatic agent, appears not to be oxidized to catechol by bacteria; its chemical configuration makes it likely that this would also hold true for mammalian tissues.

On the other hand, it has been known for many years that in man administered salicylate is oxidized in part to gentisate(16). The possibility exists, therefore, that gentisate or some oxidation product of gentisate could be

ultimately responsible for the antirheumatic activity of salicylate. Therefore, the finding by the method of simultaneous adaptation that gentisate is not oxidized through any pathway known for other aromatic substances, *e.g.*, via catechol, protocatechuate, or homogentisate, introduces the necessity of searching out other likely intermediates, including straight-chain keto-acids. Indeed, if the oxidation of gentisate were quite analogous to that of homogentisate one would expect the production of a β-diketo-, dicarboxylic acid, $C_7H_6O_6$. The qualitative chemical tests carried out so far indicate that such a reaction is entirely possible.

A search through the chemical literature has failed to turn up any helpful information on this score: fumarylpyruvic acid may exist but it has not been synthesized or isolated from natural sources, and must therefore remain for the time being a hypothetical intermediate in the oxidation of gentisate. The problem of the isolation and identification of the oxidation products in the bacterial system must next be undertaken. It may later be possible to apply the findings to a study of gentisate oxidation in man.

Summary. 1. The oxidation by bacteria of salicylate, gentisate, and 2:3-dihydroxybenzoate has been examined with the technic of simultaneous adaptation. 2. Catechol appears to be an intermediate in the oxidation of salicylate. 3. Organisms grown on gentisate and 2:3-dihydroxybenzoate show no adaptation to any likely known intermediates. 4. It is suggested that the oxidation of gentisate may be analogous to that of homogentisate, with formation of a straight-chain β-diketo-dicarboxylic acid.

The authors also wish to thank Drs. K. Meyer and C. Ragan for many helpful suggestions and some of the chemicals, and Dr. J. MacLennon for advice in bacteriology.

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Glucosamine and Leukemia.* (20534)

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Recently Quastel and Cantero(1) reported an inhibition of mouse sarcoma 37 by glucosamine. The rationale of their study was as follows. Hexokinases are able to phosphorylate glucosamine *in vitro*(2,3), the reaction being competitive with glucose. Tumors, presumably being particularly dependent on glycolytic energy sources, might be selectively influenced by a glucosamine block of glucose

* Supported in part by the P. C. Pinson Fund.

TABLE I. Clinical and Hematological Data.

Case:		1*	2†	3	4	5
Age – Sex:		9 – ♀	24 – ♂	28 – ♂	62 – ♀	68 – ♂
Diagnosis:		Acute lymphocytic	Acute histiomonocytic	Chronic myelocytic	Chronic lymphocytic	Chronic lymphocytic
Glucosamine dosage (g/24 hr):		3 days— 1.5 3 " — 3.0 1 " — 4.0 24 " —10.0	1 day— 3.0 3 " — 9.0 2 " —15.0 16 " —20.0	5 days— 6.0 8 " —10.0	1 day— .5 2 " —1.0 1 " —2.0 1 " —3.0 1 " —4.0 3 " —5.0	1 day— 3.0 1 " — 5.0 8 " —10.0
Hb, g/100 ml	Before	11.7	10.5	12.7	13.8	12.4
	After	8.0	11.5 ‡	12.5	11.9	12.2
RBC × 10^6	Before	4.36	3.70	4.49	3.86	4.16
	After	2.85	3.80‡	4.48	3.79	4.26
WBC × 10^3	Before	113	5	29	22	39
	After	298	72	23	23	44
Plats. × 10^3	Before	148	150	98	88	194
	After	80	100	84	96	170
Differential	Before	Blasts. —25.0 Lymphs.—71.0 Neut. — 4.0	Blasts. — 6.0 Monocytes—65.0 Neut. — 4.0 Lymph. —25.0	Blasts.— .5 Lymph.–3.0 Monos.—1.5 Immat. granulocytes—36.0 Mat. granulocytes —59.0	Lymphs.—86.0 Neut. —14.0	Lymphs.—88.0 Monos. — 2.5 Baso. — .5 Neut. — 9.0
	After	No change	No change	No change	No change	No change
Bone marrow	Before	Crowded with blasts	Crowded with blasts—apparent differentiation into monocytes	Permission refused	Mature lymphs. 80.5%	Mature lymphs.—95.1%
	After	Permission refused	No change	" "	No change	No change
Results:		Alive but condition deteriorating 2 mo. after glucosamine was discontinued.	Died 2 days after glucosamine was discontinued. Autopsy.	No change	Died 5 wk after glucosamine was discontinued. Autopsy.	" "

* Courtesy of The San Diego County Hospital, San Diego, Calif., Dr. W. J. Tighe, Chief of Medicine.
† Courtesy of The U. S. Naval Hospital, San Diego, Calif., Capt. A. L. Lawler, M. C., U. S. N., Chief of Medicine.
‡ Multiple transfusions given.

utilization and concomitant diversion of adenosine triphosphate.

Regardless of the mechanism, there was a striking inhibition of the rate of growth of the mouse tumor which was associated with convincing morphological cellular damage and an increase of liver catalase over non-treated controls. In view of these results it was decided to test the effect of glucosamine in human leukemia.

Results and discussion. Glucosamine was purchased from Eastman or prepared from local lobster shells(4). Both preparations were purified by repeated charcoal treatment and crystallization from aqueous alcohol and from water. The resulting snow white crystals gave theoretical reducing values. The compound was administered orally in 0.5 g capsules in divided doses, starting with 1-2 g a day and increasing in 3 or 4 days to as much as 20 g per day. Table I summarizes the hematological findings and the dosage administered.

Except for occasional mild gastric irritation there was no demonstrable toxicity referable to the glucosamine itself. The metabolism of the compound is poorly understood. It has not been found in the urine after large doses in man(5). We were not able to demonstrate it in the urine in significant amount as reducing material, although it possibly was present in conjugated form.

In this limited series of cases there was no favorable influence of the glucosamine on the course of the leukemia.

It should be emphasized that a highly purified glucosamine was employed in this experiment. As a hydrolytic product of the polysaccharide, chitin, it is not unlikely that impure products contain variable amounts of hexosamine polymers. It is also conceivable that such polymers might possess oncolytic activity. Pertinent are the cytotoxic effects of small amounts of bacterial polysaccharides containing glucosamine(6,7). The early observation of glycogen inhibition of rat sarcoma(8) may also be mentioned.

A lack of effect on leukemia does not necessarily indicate that glucosamine will not influence other forms of malignancy.

Summary. Highly purified glucosamine at dose levels as high as 20 g per day for several days did not influence the course of one patient with acute lymphocytic, one with acute histiomonocytic, one with chronic myelocytic and 2 with chronic lymphocytic leukemias.

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Quantitative Assay of Melanophore-Expanding Property of Preparations of ACTH and Corticotropin B. (20535)

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In the past the melanophore-expanding property of anterior pituitary and median lobe preparations has been estimated in frogs or on excised frog skin and was attributed to a hormone termed "intermedin"(1,2). Recent studies have cast doubt upon the existence of "intermedin" as a separate entity and it has been suggested that ACTH and "intermedin" are identical(3,4). The present experiments demonstrate that the melanophore-expanding activity of preparations of ACTH can be quantitatively assayed on the amputated tadpole tail. Among the several preparations used in studying this procedure was Corticotropin B, previously shown to exert potent melanotropic and ascorbic acid depleting activity(5).

Materials and methods. Tadpoles (*R. clamitans*; 4 mm hind limb stage) were obtained from a commercial source and maintained in spring water in white enameled pans. To assure maximal contraction of the tail skin chromatophores, the tadpoles were exposed to artificial white light for 9 hours per day for a period of at least 3 days. A diet of boiled spinach was supplied daily.

Weighed samples of the ACTH preparations to be assayed were diluted to the appropriate concentration in amphibian saline (0.7% NaCl) and immediately placed in small covered Petri dishes. The Provisional USP Corticotropin Reference Standard was used as a standard for melanophore-expanding activity and was arbitrarily assigned a melanophore-expanding potency of 0.005 unit per mg. All results have been expressed in terms of this arbitrary unit. The USP Corticotropin Standard was diluted to contain 0.08 to 0.0009 melanophore-expanding unit per ml. Within this concentration range the log dose-response curve was essentially linear.

The tadpole tails were amputated and immediately immersed in the test solutions for 30-40 minutes. They were then removed, gently blotted and scored under the microscope (100x) for the degree of melanophore-expansion. The scoring procedure was patterned after that of Langrebe and Waring (6,7). In this system the unexpanded melanophore was assigned a score of 1; the fully expanded, multipolar structure was assigned a score of 5. Intermediate scores (2, 2½, 3, 3½, etc.) were given to the partially expanded, asteroid melanophores depending upon their degree of extension. This procedure is one of reasonably satisfactory precision under the conditions employed. According to Thing(8) measurements of melanophoric expansion obtained with a photoelectric cell yielded no better results than subjective scoring.

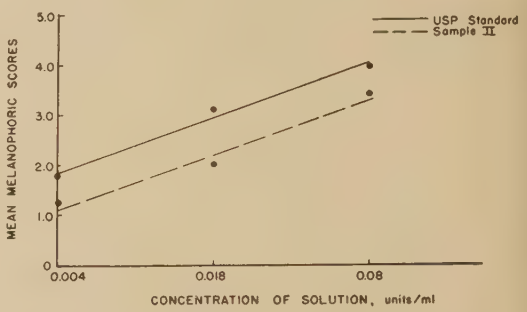

FIG. 1. Dose response curves; USP corticotropin Standard and Sample II (Tables I and III).

Results. Fig. 1 shows the log dose response curves plotted from the data (melanophore indices) recorded in Table I. The Analysis of Variance (Table II) indicates that these 2 dose response curves are essentially parallel and that the slight curvature is not significant. The potency of the unknown relative to the standard, calculated by the procedure given by Bliss(9), was 58 ± 12 units per mg.

The results of 6 assays performed by this method are shown in Table III.

Summary. Skin melanophores of freshly amputated tadpole tails expand when placed in solutions of ACTH. An assay for rapid estimation of this melanophore-expanding activity of ACTH preparations has been de-

TABLE I. Melanophoric Indices Observed in Isolated Tadpole Tails; 35 Min. of Immersion (Avg of 2 Readings per Tail).

Conc., units/ml	Standard	Unknown
.08	3.75	3.50
	4.00	3.25
	4.00	4.00
	4.00	2.75
	4.00	3.50
	$S_H = 19.75$	$U_H = 17.00$
.018	3.50	2.00
	3.25	2.00
	2.75	1.75
	3.00	2.75
	3.00	1.50
	$S_M = 15.50$	$U_M = 10.00$
.004	2.25	1.50
	2.25	1.00
	2.00	1.00
	1.50	1.75
	1.00	1.00
	$S_L = 9.00$	$U_L = 6.25$

TABLE II.
Analysis of Variance (Melanophoric Indices).

Variation due to:	df	ms
Samples	1	4.03*
Slope	1	23.11†
Parallelism	1	—
Curvature	2	.26
Error	24	.16

* Highly significant source of variation.
† Very highly significant source of variation.

veloped, using USP corticotropin as the Reference Standard. All ACTH preparations tested showed this activity. Corticotropin B, the most highly purified preparation available, showed the highest degree of melanophore-expanding activity and was also the most potent in causing adrenal ascorbic acid depletion.

TABLE III. Melanophoric Potencies of 5 Corticotropin Preparations (Units/mg).

Sample	Melanophoric potency	Ascorbic acid depletion potency
I	9 ± 5	5
II	58 ± 12	160
III	58 ± 12	60
IV	21 ± 5	60
V	10 ± 3	5
VI*	269 ± 48	163 ± 141

* Sample of Corticotropin-B.

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Effect of Adrenal Cortical Extract and Steroids upon Glucose Tolerance of Eviscerated Rats. (20536)

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The data of this study support the conclusions of an earlier report(1) that adrenal cortical extract inhibits the tolerance of the eviscerated rat for intravenously administered glucose. Cortisone and hydrocortisone are less effective.

Methods. General details have been published(1,2). Male rats were eviscerated at 250 ± 2 g weight. Each rat received by continuous intravenous injection 20 cc per 24 hours of a solution containing glucose, 4 units of regular insulin, 10,000 units of penicillin

TABLE I. Effect of Adrenal Cortical Hormones upon Level of Blood Glucose in Eviscerated Rat. Averages and standard errors.

Exp.	Duration, hr	Cortical hormone	Dose, 24 hr	Glucose load, mg/100/hr	No. rats	Blood glucose, mg %
1	24	Controls	0	40	12	91 ± 9.8
	24	ACE	20 cc	40	12	141 ± 16.4*
	24	Controls	0	44	12	86 ± 12.9
	24	ACE	20 "	44	12	168 ± 29.0*
	24	Controls	0	48	12	164 ± 20.1
	24	ACE	20 "	48	12	251 ± 30.1*
2	24	Controls	0	44	52	110 ± 5.8
	24	Cortisone	4 mg	44	52	127 ± 8.0
	24	Hydrocortisone	4 "	44	52	128 ± 8.2
3	48	Controls	0	44	35	187 ± 15.6
	48	Cortisone	4 "	44	36	191 ± 15.0
	48	Hydrocortisone	4 "	44	35	223 ± 16.1
4	24	Controls	0	44	24	105 ± 8.1
	24	ACE	20 cc	44	24	174 ± 17.2*
	24	Hydrocortisone	2 mg	44	24	109 ± 4.8

* The difference between this avg and that of the corresponding control group meets the usual requirement for statistical significance.

and 5 mg of streptomycin with and without adrenal cortical extract (ACE) or non-esterified steroids. The ACE was derived from mixed hog and beef adrenal glands and represented 20 glycogen units(3) per rat per 24 hours which is the activity equivalent of 2 mg of hydrocortisone. Temperature was constant at 26.5 ± 0.5°C. The glucose load is expressed as mg of glucose per 100 g of rat per hour (mg/100/hr). The level of blood glucose was determined(4) on jugular vein blood at the end of the experiment.

Experiments and results. The data are in Table I. Comparisons of rats with and without adrenal cortical hormones were made simultaneously in each experimental group. The administration of ACE was associated with a significant elevation of the level of blood glucose above that of the controls (Exp. 1 and 4); each of the 2 steroids failed to cause a significant elevation in the level of blood glucose under these conditions (Exp. 2, 3, and 4).

Discussion. ACE can affect the glucose tolerance of the eviscerated rat given insulin but cortisone and hydrocortisone are either ineffective or relatively weak in this respect. In the non-eviscerated rat the diabetogenicity and the glycogenic potency of 4 mg per day of either steroid is much greater than that of 20 cc of ACE. Does the effect of ACE upon the glucose tolerance of the eviscerated rat represent some physiological function of the cortical hormones? Is this effect due to the presence in ACE of some hormonal principle other than either cortisone or hydrocortisone, or is it due to some non-hormonal contaminant? The results of this study may relate to the observation(5) that adrenal cortical extract modifies the hexokinase reaction, whereas cortisone fails to do so. It has been considered possible that these steroids undergo some bio-conversion in the liver before they become physiologically active, yet, each of these steroids, as well as ACE, has a positive effect upon the ability of the adrenalectomized-eviscerated rat to work(6) and cortisone will raise the level of plasma amino acids in such animals(7). The biological responsiveness of the eviscerated rat to adrenal cortical hormones is, in our experience, sluggish and feeble as compared to that of non-eviscerated rats. The role of liver in adrenal cortical physiology is not clear.

Summary. Eviscerated rats were each given continuous intravenous injections of 20 cc per 24 hours of solutions containing glucose, insulin, and antibiotics with and without adrenal cortical hormones. Adrenal cortical extract caused a significant elevation of blood

glucose above the average value for control animals but cortisone and hydrocortisone were relatively ineffective.

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Plasma Radioactive Iron Turnover in Acute Viral Hepatitis. (20537)

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The literature contains many references to the hypersideremia of acute hepatitis(1), and in the past year additional papers have appeared(2-6). Histochemical studies of liver biopsy sections have revealed an increased deposition of "stainable-iron" in the liver in acute hepatitis(7-9). None of the studies have to date been concerned with an attempt to locate the responsible metabolic defect. Previously proposed theories attempting to explain the disturbance have been either contrary to accepted theories of iron metabolism, or have lacked any strong evidence in their behalf(1). The aberration in iron metabolism may result from a disturbance in the function of: (a) deposition (storage) of iron in the liver, (b) utilization of iron (red cell production), (c) excretion, or (d) absorption of iron.

In this study, the turnover of intravenous radioactive iron^{59} has been made in 8 patients with acute viral hepatitis. Similar studies in 5 normals, one patient with refractory anemia, and one patient with hemolytic anemia have been included for comparison.

Methods. The studies were carried out in a manner similar to the procedure described by Huff(10,11), except that the iron was made up in 2% sodium citrate. Between one to 6 μc and 5 to 30 μg of iron was used. The procedure was carried out in the morning after a 12-hour fast. Serum iron samples were taken at the time of injection and analyzed for non-radioactive iron(12). Samples of plasma and

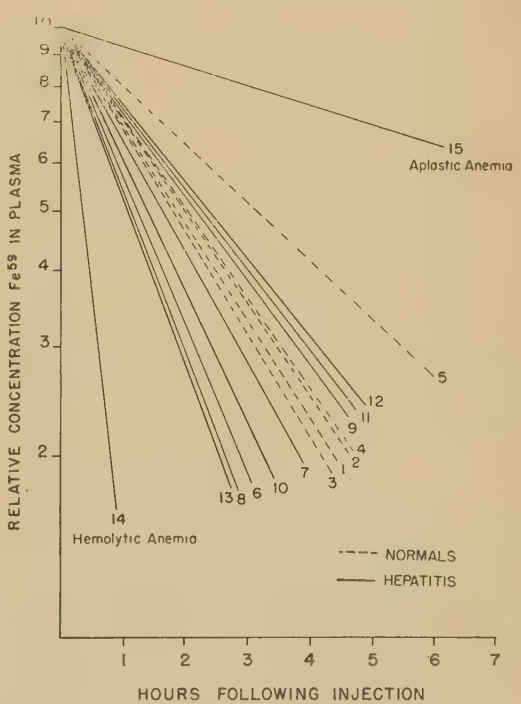

FIG. 1. Disappearance radioactive iron^{59} from plasma following intravenous injection.

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red cells were analyzed for iron^{59} by a procedure described previously(13). Tests of liver function were carried out by methods previously referred to(1). Blood samples were taken ½, 1½, 2½, 3½, and 4½ hours after injection iron^{59} and the radioactivity per ml plasma determined. Plasma volumes were determined by extrapolating the curve of plasma clearance of injected iron^{59} to zero time, and dividing the concentration of iron^{59} at this point into the amount of iron^{59} injected. Blood was taken at 4, 7, 10, and 15 days for determination of the iron^{59} incorporated into the red cells. Counting rates per ml plasma were plotted versus time on semilogarithmic paper (Fig. 1). The rate of removal of iron^{59} from the plasma (plasma iron rate constant) was computed by dividing the natural logarithm of 2 by the half time of disappearance as obtained from Fig. 1. The mg plasma iron turned over per day, per kilo body weight was determined as follows (Fig. 2):

$$\frac{(\text{plasma Fe rate constant}) \times (24\text{ hr}) \times (\mu\text{g Fe/ml plasma}) \times (\text{plasma vol})}{(1000\ \mu\text{g/mg}) \times \text{wt kilos}}.$$

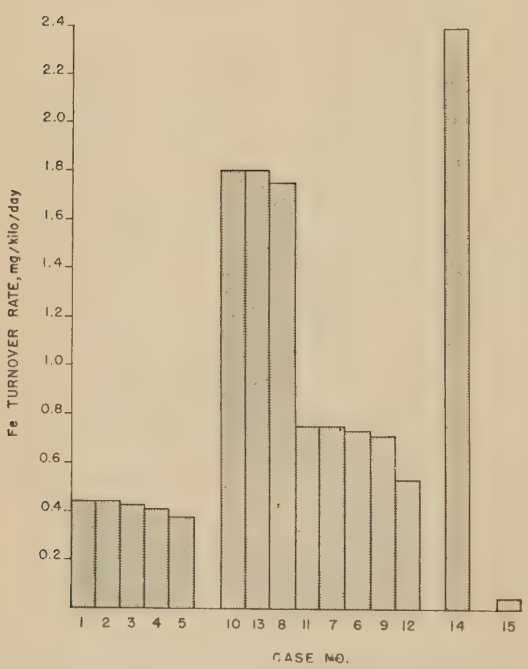

FIG. 2. Plasma iron turnover rate, mg/kilo/day.

The iron turnover in the red cells (mg/kilo/day) was computed as follows (Table I):

$$\frac{\text{Fe}^{59}\text{ red cells}}{\text{Fe}^{59}\text{ injected}} \times \text{plasma Fe turnover.}$$

The fraction of the red cell iron turned over per day (% red cell mass turned over per day, Table I) was determined by first multiplying the red cell volume by the amount of iron contained in one ml red cells (1 mg Fe/ml packed red cells).

Results and discussion. In 4 of the 8 cases (Nos. 8-11) the turnover studies were done at the time of maximal sideremia (usually at a point when the serum bilirubin begins to fall(1)). In Case No. 7 the study was done at the time of maximal bilirubinemia and at a time when the patient had a normal serum iron, but subsequently developed a mild hypersideremia. Patient No. 6 was studied prior to the time of maximal bilirubinemia and sideremia, but also subsequently developed a mild hypersideremia. Case No. 12 was studied on the 25th day after the onset of icterus, and 19 days following maximal bilirubinemia, at a time when the serum iron and bilirubin were only slightly elevated. Case No. 13 had a long-standing recurrent "cholangiolitic" hepatitis with a serum bilirubin maintained around 9 mg %, and on occasion was found to have a hematocrit as low as 38%.

Plasma iron rate constant. In the patients with hepatitis there was considerable variation in the rate constant and in the half-time of disappearance of the iron (Fig. 1); however, in 4 of the 8 cases these values were greater than the normals. In the 5 normals the plasma iron turnover rate constant was around 0.35, except in No. 5 with a value of 0.22. This was associated, however, with a high normal serum iron level.

Plasma iron turnover rate. The plasma iron turnover rate in terms of mg per kilo per day averaged 0.42 mg in the normals (26 to 37 mg per day). In the patients with hepatitis the turnover in mg per kilo per day was in all cases higher (40 to 115 mg per day) than the normal range (Fig. 2). The much higher rates of iron turnover in these patients in the absence of greatly increased turnover

TABLE I. Radioactive Iron Turnover Studies.

Case No.	Plasma Fe, μg/ml	Plasma vol, L	Red cell vol, L	Red cell Fe turnover, mg/kg/day	% red cells turned over/day	Day icterus	Max. serum bilirubin
				Normals			
1	1.4	3.0	2.8	.37	1.11		Normal
2	1.5	2.5	2.2	.40	1.22		"
3	1.3	3.0	2.9	.30	.86		"
4	1.2	2.6	2.3	.33	.92		"
5	1.9	3.0	2.6	.34	1.04		"
				Acute hepatitis			
6	1.5	2.5	2.6	.51	1.34	5	
7	1.7	3.0	2.8	.63	1.54	8	12.0
8	3.2	2.5	2.2	1.67	5.00	10	7.1
9	2.4	2.3	1.8	.57	1.83	11	6.5
10	3.3	2.6	3.2	1.35	2.40	12	32.0
11	3.1	2.7	2.4	.60	2.04	13	11.0
12	2.2	2.6	2.5	.49	1.44	25	29.0
13	2.8	2.5	2.0	1.70	5.00		9.6
				Acquired hemolytic anemia			
14	1.2	2.8	1.2				
				Refractory anemia			
15	2.6	2.9	1.5	.04	.17		

The blocked-in figures are calculated on the assumption that iron[59] cleared from the plasma goes directly to erythrogenic sites.

rate constants and disappearance times is the consequence of the higher serum iron levels.

Red cell uptake. In the normals and the patients with hepatitis there was no significant difference in the % of the administered iron that was taken up by the red cells at 10 to 15 days, and no measurable difference in the speed of uptake. In both groups between 70 to 95% was utilized for the production of hemoglobin after 2 weeks.

This study indicates that the iron is cleared from the plasma of patients with acute hepatitis faster than normally, and that more than 70% of the iron cleared appears in the red cells in 10 to 15 days. Three possible explanations for this rapid turnover appear likely:

1) *Increased red cell (hemoglobin) production.* Following intravenous administration of iron[59] there is a maximum concentration in the marrow after one day(14). Presumably all of this iron will be released from the marrow and appear in the peripheral blood by about 2 weeks—70 to 95% of the intravenously administered dose in normals(15,16). Therefore, the rate of clearance of iron from the plasma is presumed to be an index of the rate of red cell production(10,11,17-19), *viz.*: (a) conditions that inhibit erythropoiesis slow iron turnover—irradiation, nitrogen mustard, increased alveolar oxygen tension, aplastic anemia, (b) conditions associated with increased red cell production increase iron turnover—altitude and primary polycythemia, and hemolytic syndromes. In the patients with hepatitis more than 70% of the plasma iron cleared was used for hemoglobin production—0.49 to 1.7 mg per kilo per day (Table I). If this rapid turnover rate of plasma iron is the result of an increased utilization of iron for hemoglobin production, then a greater than normal % of the red cell mass is renewed per day—1.34 to 5% (Table I). In the presence of a constant hematocrit (red cell mass) this would indicate an increased rate of red cell destruction, and thus a shortened red cell survival time.

2) *Increased clearance by the liver, or some other tissue or iron "pool".* These data do not conclusively establish that all of the iron cleared from the plasma in the first day and eventually used for hemoglobin production (70-95%) went directly to the bone marrow. It might be postulated that there is a greater than normal clearance by the liver (non-erythrogenic), and a slower release to the

marrow, but not slow enough to depress the rate of uptake in the red cells, or the final concentration in the red cells(20).

3) *Increased excretion.* Analyses of the stools (3 to 4 days) and urine (24-48 hours) were made following the injection of the iron(21). In the normals and hepatitis cases the excretion of the iron^{59} in the gut was infinitesimal (0.15-0.35% of the injected dose). In most of the hepatitis cases the urinary excretion was 2 to 10 times normal (normal—0.01 to 0.05% of the injected dose). However, this represented only a trace of the amount administered and could not have accounted for the increased rate of disappearance of iron^{59} from the plasma.

Iron-binding capacities of the sera as done by a modification of the procedure of Rath and Finch(22) have failed to show greatly increased saturation of the iron-binding protein in these patients with hepatitis, and thus there should have been no failure of the injected iron^{59} binding to the iron binding globulin.

Summary. Radioactive iron^{59} injected intravenously disappears from the plasma of patients with hepatitis faster than normally. Seventy to 95% of this administered iron appears in the circulating red cells in 2 weeks. This increased plasma clearance of iron is not the result of increased excretion of the radioactive iron by the kidneys or gastrointestinal tract. It is suggested that this increased plasma iron turnover may be related to increased red cell production.

The author is grateful for the clinical facilities extended to this project by Dr. Victor Sborov, Director of the Department of Hepatic and Metabolic Diseases.

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Experimental Histoplasmosis, I. Methods for Production of Histoplasmosis in Dogs.* (20538)

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The production of histoplasmosis in dogs by respiratory routes of infection has not been reported. De Monbreun(1), who first described histoplasmosis in dogs, reproduced the disease in 3 puppies by the intraperitoneal inoculation of the yeast phase of *Histoplasma capsulatum* in 2 and citrated blood from an infected dog in the third. Feeding of mycelial and yeast phase mixtures produced no marked clinical disease although evidence of infection was observed histologically in 6 dogs. Ruhe and Cazier(2) produced the disease in 4 out of 5 dogs by the severe and unnatural procedure of inoculation of yeast cells directly through the thoracic wall into the lung parenchyma. Cross(3) was unable to produce fatal disease by the intratracheal, oral, intramuscular, or intravenous inoculation of yeast phase *H. capsulatum*. No gross lesions were seen at necropsy of inoculated animals although lesions containing organisms were later demonstrated microscopically.

It is the purpose of this report to describe the comparative effect in dogs of the yeast and mycelial phases of *H. capsulatum* introduced by the respiratory or gastrointestinal routes and the effect of cortisone on infection.

Materials and methods. The 45 dogs used in these experiments were vaccinated against canine distemper. During a pre-inoculation period of at least 2 weeks, all dogs were found to have negative chest x-rays, histoplasmin skin tests and collodion agglutination tests(4). Baseline cultures of blood, urine, feces, gastric washings, and biopsied mesenteric lymph nodes were negative for fungi. Clinical examinations were continued throughout the observation period. Each of the 19 dogs in Exp. I was inoculated (9 gastric and 10 tracheal) with a total of 8 ml of packed mycelial growth.† The first inoculation of one ml was repeated on the 35th day and was followed by 2 ml doses on each of the 75th, 77th, and 79th days. The inocula for 9 dogs (4 gastric and 5 tracheal) and that for 10 dogs (5 gastric and 5 tracheal) were suspended in 5% hog gastric mucin(5) and physiologic saline, respectively. All inocula in these studies were suspended in 3 ml of the respective menstruums.

Each of 16 dogs in Exp. II received 2 ml of packed yeast phase *H. capsulatum*. Eight dogs were inoculated with the organism suspended in saline (4 gastric and 4 tracheal) while the remaining 8 were inoculated with the mucin suspension (3 gastric and 5 tracheal). A third experiment with 18 dogs was designed to study the possible effect of cortisone on infection. Eight animals which had been inoculated with mycelial phase 11 months previously (survivors of Exp. I) were considered to be potentially "immune"; the remaining 10 were healthy dogs not previously inoculated. All 18 dogs received intramuscular injections of 100 mg of cortisone for 16 days. On the 5th day of cortisone administration 16 (8 "immune" and 8 "susceptible") of the 18 dogs were inoculated by the same route and with the same phase of the organism suspended in the same vehicle (saline or 5% mucin) as when they were part of the first experiment. Eight of the 10 "susceptible" dogs were inoculated in the same manner as the 8 "immune" animals. The 2 remaining dogs served as non-infected cortisone treated controls (Table I). Eight of the dogs which survived this latter procedure (6 "immune" and 2 "susceptible") were subsequently chal-

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† Strain G-13, a recent isolate from spontaneous histoplasmosis in a dog, kindly supplied by Charlotte Campbell of the Army Medical Center, Washington, D.C.

TABLE I. Comparative Results of Pathogenicity for Dogs of Mycelial and Yeast Phase *H. capsulatum*.

Route and vehicle	Exp. 1 (mycelial)	Exp. 2 (yeast)	Exp. 3 (mycelial + cortisone)		Exp. 4 (yeast)	
			"Immune"§	"Susceptible"	"Immune"‖	"Susceptible"
Gastric (saline)	0/5*	0/4	0/2	0/2	1/1	
" (mucin)	0/4	0/3	0/2	0/2	2/2	
Tracheal (saline)	0/5	4/4	0/2	1/2†	0/1‡	1/1
" (mucin)	0/5	5/5	0/2	2/2	2/2	1/1
0/2 controls (cortisone)						

* Numerator = No. of dogs with fatal histoplasmosis. Denominator = Total No. inoculated.
† Both dogs acutely ill; spontaneous recovery in one.
‡ Dog acutely ill; became chronic.
§ Previously employed in Exp. 1.
‖ " " " " 1 and 3.

lenged with 2 ml packed yeast cells given intratracheally (Exp. 4).

The dogs inoculated by the gastrointestinal route received the inoculum through a stomach tube. Saline or mucin equal to the volume of inoculum was used to wash adhering material from the sides of the tube into the stomach. The dogs receiving the organisms by the respiratory route were inoculated intratracheally following anesthesia by intravenous injection of sodium thiopental. The dog's head was raised, the mouth opened, and the tongue grasped and pulled forward. A 6-inch, 18 gauge luerlock cannula was inserted through the glottis deep into the trachea. The inoculum was injected into the trachea after attaching a syringe to the cannula. The cannula was then withdrawn and the head was held high for about a minute to allow the material to drain into the bronchi. Cultures of gastric contents, urine, feces, and blood, complete blood counts and collodion agglutination tests were performed weekly for one month after inoculation, biweekly during the second month and monthly during the remainder of the observation period. The detailed hematologic and serologic data will be presented in a separate report. Complete necropsies and postmortem cultures were performed on all dogs which died or were sacrificed.

Results. Exp. 1 (Mycelial phase). None of the 19 dogs inoculated with mycelial phase *H. capsulatum* manifested signs of disease during a 6-month observation period following the primary inoculation regardless of route or menstruum employed (Table I). The 10 dogs inoculated intratracheally developed positive histoplasmin skin tests within one to 3 months. Only one of the 9 dogs inoculated by the gastrointestinal route developed a skin test reaction. Repeated antemortem cultures, as well as postmortem cultures from dogs sacrificed 6 months after the primary inoculation were all negative.

Exp. 2 (Yeast phase). All 9 dogs which received the yeast phase intratracheally developed acute progressive disseminated histoplasmosis in 3 to 5 days and died in from 11 to 42 days following inoculation (Table I). Pyrexia of 1 to 3°F, hyperpnea and partial to complete anorexia were noted 3 to 4 days post-inoculation. Paroxysmal coughing and dyspnea were observed in all animals during the course of the disease. Hyperpnea and dyspnea became more severe as the disease progressed. *H. capsulatum* was isolated from all 9 dogs from both antemortem blood cultures and from the tissues aseptically collected at necropsy. The fungus was isolated frequently from the lung, spleen, and liver, and less often from the mesenteric nodes, bronchial nodes, and heart blood. Extensive pulmonary lesions present in all 9 dogs included discrete cream colored nodules measuring 1 to 3 cm in diameter or complete consolidation of entire lobes, cavitation, liquefaction, emphysema and fibrinous pleuritis (Fig. 1). Granulomatous inflammation of the visceral lymph nodes and liver which were culturally positive for *Histoplasma* presented further evidence of widespread dissemination of the infection. None of the 7 dogs inoculated orally with yeast phase manifested disease during a 6-month post-inoculation period or became sensitive to histoplasmin. Of the 5 dogs sacri-

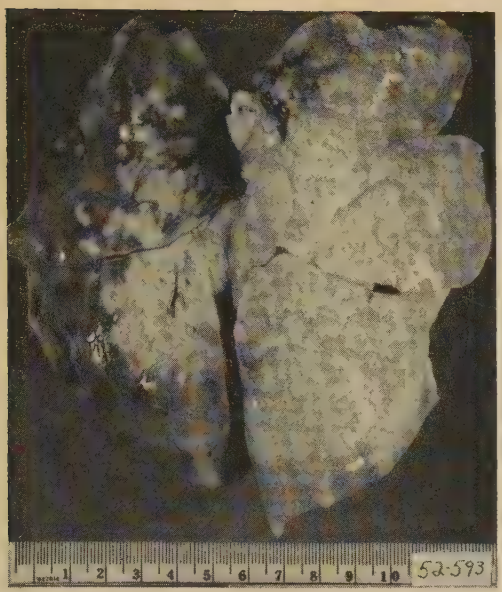

FIG. 1. Lungs (frontal section) from dog which received yeast phase *H. capsulatum* intratracheally. Consolidation of right lung, atelectasis, diffuse and nodular granulomatous pneumonia in left lung.

FIG. 2. Lungs from dog which received mycelial phase *H. capsulatum* intratracheally and cortisone. Elevated nodules in all lobes, enlarged bronchial nodes and fibrinous pleuritis.

ficed from this group no evidence of infection was found at necropsy.

Exp. 3 (Mycelial phase plus cortisone). The weight of each dog in this group increased and fluctuated after a week on cortisone treatment due, in part, to changes in the fluid balance. All 4 "susceptible" dogs which were inoculated intratracheally became ill on the 5th, 6th, 12th, and 18th post-inoculation day, respectively. Increased bronchial sounds, dyspnea, and pyrexia were the most consistent signs. Three of the 4 dogs died of acute, progressive, disseminated histoplasmosis between the 18th and 28th day after inoculation with lesions similar to those seen in Exp. 2 (Fig. 2). One developed severe illness, a high histoplasma antibody titer, positive blood cultures, but gradually recovered spontaneously. In contrast, none of the 4 "susceptible" dogs on cortisone which received the mycelial growth by stomach tube showed any signs of histoplasmosis during a post-inoculation period of 2½ months. Similarly negative results were obtained with the 8 "immune" dogs regardless of route of inoculation (Table I). The 2 uninoculated control dogs receiving cortisone only, failed to show signs of infection during this same period.

Exp. 4 (Intratracheal challenge with yeast phase). Eight dogs which had been previously unaffected clinically following inoculation with mycelial phase (Exp. 1) were not affected when challenged with mycelial phase plus cortisone (Exp. 3). To investigate further their relative state of resistance, 6 of these dogs were inoculated with 2 ml of yeast phase organisms, intratracheally. This procedure was followed by severe signs of infection in all 6 with 5 deaths occurring between the 6th to 49th days. Similar fatal infections were obtained with 2 dogs which were unaffected by gastric administration of mycelial phase in Exp. 3 when given yeast phase intratracheally (Table I); both died 10 days after inoculation.

Discussion. H. capsulatum may be grown in either the yeast or mycelial phase. It appears as a yeast in parasitized tissues. The mechanism of natural infection is not yet definitely known. Since severe fatal infections were produced only after intratracheal inoculation, it is possible that the respiratory route is more common, or likely, in spontaneous infections. That the organism was disseminated from the primary pulmonary site of infection, was demonstrated by isola-

tion of the fungus from liver, spleen, mesenteric nodes, heart blood, as well as from the lungs and bronchial nodes. Detailed description of the gross and microscopic pathology, clinical and laboratory data, including serologic results, will be reported in a separate communication.

Little is known about immunity, or reinfection in histoplasmosis. In these studies it has been demonstrated that although intratracheal inoculation with mycelial phase induced no clinical disease a relative immunity to subsequent challenge to mycelial phase, plus cortisone, was observed. However, challenge with yeast phase did produce a severe disease. Since this latter inoculum was large, similar studies with lesser dosages are indicated to clarify the nature of immunity in histoplasmosis.

Summary. Yeast phase *H. capsulatum* induced acute, progressive, fatal histoplasmosis in all of 9 dogs when inoculated intratracheally, but no clinical disease in all of 7 receiving the same inoculum by stomach tube. Mycelial phase produced no clinical evidence of infection in 9 and 10 normal dogs receiving intragastric and intratracheal inoculations, respectively. Cortisone treated dogs, however, were susceptible to intratracheal but not intragastric mycelial phase inoculations. Dogs previously receiving mycelial phase were resistant to reinfection with the same phase even when given cortisone; they succumbed to challenge with intratracheal yeast phase in the absence of cortisone.

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Transfusion of Separated Leukocytes into Irradiated Dogs with Aplastic Marrows. (20539)

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The consistent finding of bone marrow aplasia and agranulocytic ulcerations in dogs dying from lethal doses of x-ray suggested a causal role of neutropenia in death from radiation injury(1,2). To test this thesis, concentrated suspension of homologous white blood cells were transfused into irradiated leukopenic dogs. The method used was not successful in maintaining a normal level of circulating granulocytes. However, significant elevations of the white blood cell counts in dogs with totally aplastic marrows were obtained. This report describes preliminary results on the functional activity and fate of the transfused granulocytes.

Material and methods. Dogs were exposed to whole body irradiation of 600 r (cobalt 60 source, measured in air by a Victoreen rate meter in 4 π geometry), a uniformly lethal dose which resulted in complete bone marrow aplasia. Donor dogs were given an i.m. or s.c. injection of 0.2 to 0.5 ml of a 1:10 solution of turpentine in alcohol which generally elevated their circulating WBC level. All donor dogs were negative for the canine A hemagglutinogen(3). Donor dogs were bled from the femoral artery into siliconed 230 ml bottles, each containing 40 ml of 5% dextran and 0.5% di-sodium ethylenediamine tetraacetate (EDTA) in Hanks' solution. The dextrans† used were 2 lots (No. 237 and No. 649) that

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† Generously supplied by Commercial Solvents, Terre Haute, Ind.

TABLE I. Transfusion of Separated WBC into Irradiated Dogs.

Dog. No.	Day after x-ray	Leucocytes $\times 10^8$ inj.	Circulating leucocytes $\times 10^2/mm^3$ Before	1 hr after transfusion	4-6 hr after
510	5	102	8	18	
	6	74	6	28	49
	7	47	11	22	41
	8	52	10	22	44
	9	73	12	32	42
	10	77	3	19	
	11		12	16	
	12	46	9	12	
	13	126	3	18	
	14	57	4	8	
	15	59	7	6	4
	16	62	2	6	4
	17	52	1	4	2
	18		0.2	3	
	19	59	1	4	4
	20	69	1	2	3
518	5	94	22	34	52
	6	98	12	8	13
	7	57	2	3	4
	8	159	2	23	35
	9		6		

Martin(4,5) found to interfere but little, if at all, with WBC respiration. A third lot of dextran (No. 546) used from the 15th-20th post-irradiation day in dog No. 510 (Table I) was later found to interfere with WBC respiration. Thirty minutes were allowed for sedimentation of red blood cells. The plasma, which contained 80-90% of the leucocytes and platelets originally present in the whole blood, was siphoned through siliconed glass and polyvinyl tubing into a second set of siliconed bottles. The plasma was centrifuged at room temperature at 30x*g* for 20 minutes or 150x*g* for 30-45 minutes. This resulted in sedimentation of 70-90% of the leucocytes which were resuspended in a small amount of plasma and injected. 760 ml of whole blood were processed for each daily transfusion, yielding 30-40 ml of a suspension of 100000-300000 leucocytes per cu mm, representing 50-80% of the leucocytes originally present in the blood drawn. The cell suspensions prepared by centrifugation at 150x*g* also contained one to 5 million platelets per cu mm. Thirty to 40 ml of supernatant plasma which was poor in WBC's and platelets were injected into irradiated control dogs.

Results. In untreated irradiated dogs, the leucocytes generally fell to levels below 2000/cu mm by the 5th post-irradiation day. The WBC count remained below 1000, and usually below 500 per cu mm from the 7th day on. Four irradiated dogs receiving daily transfusions of plasma poor in leucocytes and platelets showed the same low levels of circulating cells. Four dogs received a total of 30 transfusions of WBC concentrates between the 7th and 20th post-irradiation day. Data on 20 of these transfusions are presented in Table I. The weights of the recipient dogs varied from 10 to 13.6 kg and their estimated blood volume from 800 to 1100 ml. In dog No. 518, weighing 13.6 kg, the maximum expected rise on day 5 was 9000 WBC's per cu mm, and on day 8 it was 14000 leucocytes/cu mm. The circulating cells actually rose by 3000 cells on day 5 and by 3300 cells on day 8, representing 30% and 25%, respectively, of the expected rise. In most of the transfusions, however, much smaller proportions of the transfused leucocytes circulated. Elevation of the recipient's platelet level was similar to that in previously reported experiments when platelet rich suspensions were used(6).

About 80% of the leucocytes in the concentrates were polymorphonuclear cells, and rises in circulating leucocyte levels were primarily due to increases in granulocytes. Even when

transfusions were followed by only small rises in the leucocyte count, there was a clear-cut shift from a predominantly lymphocytic differential blood picture to one with a relatively high percentage of polymorphonuclear cells.

One of the 4 dogs receiving leucocyte transfusion died on the 20th post-irradiation day. The others were killed 24 hours after the last transfusion. Dog No. 510 had developed a folliculitis during the last 10 days of life and polymorphonuclear leucocytes were found infiltrating hair follicles in sections taken at autopsy. Polymorphonuclear infiltration of a small ulcer of the tonsil was present in dog No. 518. The other 2 dogs showed polymorphonuclear infiltration of localized skin lesions. No polymorphonuclear leucocytes were seen in the lung or spleen of these dogs. Granulocytes were present in the sinuses of some of the lymph nodes, although no infectious lesions were noted in the region drained by these nodes. In contrast, none of the 4 dogs receiving WBC-poor plasma showed granulocytes in any organ of the body. Three were killed 24 hours after the last transfusion. One died on the 9th day and showed the granulocytic tonsillar lesion characteristic of dogs dying spontaneously from exposure to lethal doses of x-ray(1).

Discussion. The elevation of the peripheral leucocyte count resulting from transfusion of fresh leucocyte suspension, although variable, appears significant, since similar results have not been obtained previously in the presence of marrow aplasia, except on cross-circulation(7). In unpublished experiments, 50 dogs were exposed to 600 r whole body x-irradiation and died or were killed between the 6th and 23rd post-irradiation day and sections taken of all organs. In none of these were granulocytes seen. The presence of granulocytes in tonsillar ulcers and skin lesions of animals receiving WBC transfusions indicates that the transfused leucocytes were able to migrate to sites of infection. The accumulation of granulocytes in lymph node sinuses in the absence of anatomic evidence of infection in the region drained by these nodes suggests the possibility that lymph nodes may participate in the removal of granulocytes from the circulation in irradiated dogs. However, it cannot be excluded that the leucocytes in the lymph node sinuses represent the response to a minor local infection which may have been overlooked.

Summary. The feasibility of recirculating separated leucocytes has been demonstrated in dogs with complete bone marrow aplasia induced by x-irradiation. The transfused granulocytes were shown to migrate to sites of infection.

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Virus Growth and Cellular Energy Production: Effect of Substances Chemically Related to Thyroxin on Influenza Virus.* (20540)

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Growth of influenza virus in tissue cultures has been partially inhibited with 2,4-dinitrophenol (DNP) under conditions which indicate that high energy bonds derived from oxidative phosphorylation play a part in growth of this virus(1-3). An antagonist of thyroxin, butyl 3,5 diiodo-4-hydroxybenzoate (4), has an even more powerful action than DNP in uncoupling phosphorylation from oxidation(5,6). This suggested studies of the effect of this compound on virus growth and a comparison with the action of DNP. Thyroxin itself and other related substances have also been investigated.

Materials and methods. The PR8 allantoic passage strain of influenza virus was used in all experiments. Methods of tissue culture in flasks, and the preparation of deembryonated eggs have been described previously(3). In addition, a modification of the roller tube technic was adopted which permitted direct observation of fragments of chick embryo chorioallantoic membrane sticking to the glass walls of the tubes. The medium used was Hanks' balanced salt solution with certain combinations of sodium pyruvate and glucose. The pH was adjusted to 7.6 and maintained as close to this level as possible. No sodium bicarbonate, serum, plasma or chick embryo juice was added because the duration of the experiments was short and it was desired to employ as simple a medium as possible. The roller tubes were closed with plastic screw caps which were kept loose to permit escape of CO_2. Because of their limited solubility in aqueous media the butyl† and methyl esters of the substituted benzoic acid were sterilized by heating concentrated alcoholic solutions and then diluted 1:1000 or more in alkaline balanced salt solution. Alcohol alone at concentrations as high as 1% had no effect on the growth of virus or on proliferation of the chorioallantoic tissue. Diiodotyrosine and 3,5 diiodo-4-hydroxybenzoic acid (Eastman) were sterilized by heating the aqueous suspensions to 80°C for 20 minutes and dissolved by adding NaOH to bring the pH slightly above neutrality. At the conclusion of the experiments pooled samples of the tissue were suspended in fresh balanced salt solution with 0.1% glucose and measurements of oxygen consumption were made by the usual Warburg method.

Results. Warburg experiments were done with fresh minced chorioallantoic membrane to test for stimulation or depression of respiration by butyl 3,5-diiodo-4-hydroxybenzoate. The tissue was rinsed 3 times in balanced salt solution and suspended in this same solution containing the test substance. One set of experiments was done with 0.1% pyruvate in the balanced salt solution and a second set with 0.1% glucose. In the presence of pyruvate both 20 γ/ml and 5 γ/ml of the butyl ester produced an approximately 50% increase in the respiration within a few minutes, and some stimulation was evident, particularly with the lower concentration, after 7 to 8 hours. At 24 hours there was no significant difference in O_2 consumption between the treated and control tissue. The medium remained alkaline. In the presence of glucose, on the other hand, the butyl ester produced a rapid drop in pH, and there was almost complete inhibition of oxygen consumption within 3 to 4 hours. No initial stimulation was detected. These results are essentially the same as those previously detailed for dinitrophenol and chorioallantoic tissue(3). As with DNP a drop in the pH of the medium causes a relatively higher toxicity of the drug.

The results of experiments with influenza virus in flask and roller tube cultures are presented in Table I. The flask cultures with

* This work was aided by grants from the U. S. Public Health Service and the Eugene Higgins Trust.

† A supply of the butyl ester was furnished by Dr. A. E. Heming of Smith, Kline and French, Inc. The methyl ester was prepared according to the method of Wilkinson and his associates(4).

TABLE I. Effect of Butyl and Methyl Esters of 3,5-diiodo-4-hydroxybenzoic Acid on Growth of Influenza Virus in Tissue Culture.

Tissue culture	Ester, γ/ml		Day tested	Hemagg. titer	Final pH	Epithelial† growth	Fibroblast growth	O_2, μl/g/hr
Flask*	Butyl	8	6	<2	7.7	±	±	45
	"	4	6	4	7.9	+, ±	+	145
	"	2	6	175	7.8	+, ±	+	150
	—	0	6	265	7.7	+	+	160
Roller tube*	Butyl	5	3	<2	6.9	±	0	0
	"	2.5	3	2	7.5	+	+	140
	"	1	3	40	7.6	+	++	212
	"	0	3	215	7.5	++	++	202
	Methyl	10	3	<2	6.8	0	±	0
	"	5	3	25	7.0	+	+	73
	—	0	3	112	7.9	+	+	218
	Free acid	200	3	65	7.2	+, ±	+	N.D.
	Thyroxin	50	3	80	7.1	±	++	80

Virus inoculum 0.1 cc of 10^{-4} or approximately 10 tissue culture infectious doses. Results represent averages of duplicate experiments.

* Medium in flasks was Hanks' balanced salt solution with 0.02% glucose and 0.1% sodium pyruvate, in roller tubes 0.05% glucose and 0.1% pyruvate.

† For flask cultures growth was observed in fragments of tissue explanted to fibrin clot medium; in roller tubes growth was observed directly by microscopic examination of tissue sticking to wall of tube.

a medium containing 0.1% pyruvate and 0.02% glucose were gently shaken in a special apparatus during the period of incubation (3). The hemagglutination titer remained low for periods as long as 6 days in the presence of 4 γ/ml‡ of the butyl 3,5 diiodo-4-hydroxybenzoate. There was only slight retardation of tissue growth in explants and little depression of oxygen consumption. Concentrations of 8 γ/ml were found to be toxic in this medium. With 0.1% glucose in place of pyruvate, concentrations as low as 2 γ/ml were toxic even when $CaCO_3$ or $NaHCO_3$ were added to control the drop in pH.

In roller tube cultures 2.5 γ/ml of the butyl ester produced a hundredfold reduction in the hemagglutination titer at 3 days without noticeable effects on fibroblastic outgrowth but with slight depression of epithelial proliferation and some inhibition of oxygen consumption. The methyl ester at concentrations of 5 γ/ml produced only a slight depression of hemagglutinin formation despite rather marked inhibition of oxygen uptake. Other experiments were done with the sodium salt of 3,5 diiodo-4-hydroxybenzoic acid. This substance produced noticeable effects on virus growth only at concentrations of 200 γ/ml or higher but it caused development of acidity, probably attributable to increased glycolysis, at concentrations between 100 and 1000 γ/ml.

Wilkinson (4) has reported that the antithyroxin effect of the butyl ester is about twice that of the methyl ester and that uneserified 3,5 diiodo-4-hydroxybenzoic acid is inactive. Attempts to reverse the virus inhibitory effect of the butyl ester by adding thyroxin at concentrations of 5 to 50 γ/ml to the roller tube tissue cultures at the beginning of the experiment have so far been unsuccessful. Thyroxin by itself had little effect on the growth of influenza virus under those conditions but at concentrations of 50 γ/ml it tended to inhibit tissue proliferation and oxygen consumption after 3 days' incubation (Table I). Further studies with this hormone are in progress.

The more rapid growth of virus in deembryonated eggs made it possible to study the early effects of the butyl ester both on infectivity and hemagglutination. Little inhibition of virus was obtained below 10 γ/ml but at this concentration the ratio of the hemagglutination titers, control/treated, after 72 hours' incubation was 8 in one experiment and 70 in another. At concentrations of 20 γ/ml hemagglutinins were detectable in only

‡ This is approximately 8.5 x 10^{-6} M.

TABLE II. Effect of Butyl Ester on Influenza Virus in Deembryonated Eggs.

		Time in hr			
		18	24	48	72
Hemagg.*	Treated	<2	<2, <2, <2, 512	<2, <2, <2, 256	<2, <2, <2, 100
	Control	2, 8, 160, 160	1900	7700	2600
Infect.† (log)	Treated	4.0, 5.0	5.5	5.0	—
	Control	6.5, 7.0	8.5	7.5	—
O_2 uptake, μl/g/hr	Treated	169	200	108	75
	Control	240	240	122	180

Concentration of butyl ester 20 γ/ml; alcohol 1% by volume in this set of experiments.

* Where there were marked differences, titers of individual deembryonated eggs are presented. Elsewhere the single figure represents the avg.

† Infectivity titrations were done on pools of 4 individual fluid samples. Initial titer approximately 10^2 E.I.D. 50.

about 25% of the treated cultures. Several experiments were done at this level of the butyl ester and minced samples of the membranes from 3 to 4 preparations were pooled and tested for O_2 uptake at various periods from 18 to 72 hours. In addition the fluids were titrated for infectivity by allantoic inoculation of chick embryos. The results are summarized in Table II. In the first 24 hours an increase in infectivity titer occurred in both treated and control preparations but both hemagglutination and infectivity titers of the treated cultures remained below the controls for a period of 48 hours. During the period of maximum inhibition of virus growth the butyl ester reduced the oxygen consumption only by 15 to 30% but after 72 hours the concentration of 20 γ/ml began to produce deleterious effects as indicated by more definite depression of respiration.

Diiodotyrosine and sodium 3,5 diiodo-4-hydroxybenzoate at a concentration of 2000 γ/ml produced little or no inhibition of virus growth in deembryonated eggs but oxygen consumption after 48 hours' incubation was depressed by about 30%.

Experiments in chick embryos inoculated by the allantoic route indicated that the LD50 of the butyl ester was 2 to 4 mg per egg when given as a suspension. Because of the low solubility of the compound it is likely that only a small part of the amount injected was actually in solution in the allantoic fluid. With the maximum possible dosage no inhibition of virus growth was found at periods of 18 to 48 hours after inoculation.

Discussion. In most respects the butyl ester of 3,5 diiodo-4-hydroxybenzoic acid closely resembles 2,4-dinitrophenol(3) in its action on tissue cultures and deembryonated eggs infected with influenza virus but on a molar basis it is about 5 to 10 times as active as the latter compound in its inhibitory action both on virus and tissue. The reported low toxicity of the butyl ester for mice(4) by subcutaneous injection, 3500 mg/kg is of interest in view of the considerably higher toxicity, about 80 mg/kg, in chick embryos inoculated by the allantoic route, and the still higher toxicity in tissue cultures. Although dinitrophenol at concentrations which were effective in tissue cultures also produced temporary inhibition of influenza virus in the allantoic sac of chick embryos, this effect was not observed with the butyl ester possibly because of its insolubility or rapid destruction.

Thyroxin itself produced very little inhibition of the growth of influenza virus in tissue culture even at concentrations of 50 γ/ml which tended to depress epithelial growth and respiration. Although thyroxin has been reported to have an effect like dinitrophenol on oxidative phosphorylation(5,7) in certain mitochondrial preparations, unknown factors must prevent this uncoupling action from inhibiting the growth of influenza virus in intact cells. Delayed absorption or a latent period before activation may be concerned. The failure of thyroxin to reverse the virus inhibition suggests that the observed effects may be separate from the mechanism of antithyroxin action of the butyl ester in thyroidectomized rats(4).

Conclusion. Butyl 3,5-diiodo-4-hydroxy-

benzoate, a substance which uncouples phosphorylation from oxidation, has effects very similar to dinitrophenol in inhibiting the growth of influenza virus in tissue cultures and deembryonated eggs.

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In vivo Inhibition of Monoamine Oxidase Studied with Radioactive Tyramine. (20541)

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In an earlier publication evidence was presented for the participation of monoamine oxidase in epinephrine metabolism and for inhibition of this enzyme *in vivo*(1). Epinephrine, because of the complexity of its metabolism(2) is not an adequate substrate for extended studies of monoamine oxidase *in vivo*. A more nearly ideal substrate, tyramine, a compound widely used for *in vitro* studies on monoamine oxidase, has therefore been synthesized in radioactive form. It is shown that with the exception of a small amount of conjugation, tyramine is metabolized by rats and mice only through monoamine oxidase action.

A simple method for testing *in vivo* inhibition of monoamine oxidase has been devised and applied to a number of compounds which not only have *in vitro* inhibitory power but also have pronounced pharmacological activity. Because of the relationship of some of these compounds to the sympathetic nervous system, a clarification of their effect on monoamine oxidase in intact animals is of considerable importance. It has been proposed by Gaddum and Kwiatkowski(3) that the sympathomimetic activity of ephedrine may be due to inhibition of monoamine oxidase and consequent protection of epinephrine from destruction. However, in the present study, no relationship is found between sympathomimetic activity and *in vivo* inhibitory power for monoamine oxidase.

Isotopic compounds. Tyramine, labeled with C^{14} in the α-position of the side chain was prepared by a small scale modification of the method of Johnson and Daschavsky (4). It involves thermal decarboxylation of DL-tyrosine, the latter being purchased in radioactive form from Tracerlab, Inc. The crude tyramine was purified by sublimation and converted to the picrate. The melting point of the radioactive tyramine picrate was 201°; Barger(5) reported 200°. For a non-isotopic sample synthesized by the same procedure, calculated C 45.9, H 3.85, N 15.3; found C 46.2, H 4.12, N 15.6. The compound showed only one radioactive spot on paper chromatograms. The specific activity was 3.3×10^6 c.p.m. per mg measured in a flow counter. Tyramine was converted to the hydrochloride before injection into animals.

Metabolism of tyramine. In order that radioactive tyramine be of maximum value in these experiments it was necessary to establish the extent to which monoamine oxidase is responsible for its metabolism. Previous workers had shown that in dogs, cats and

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rabbits(6) the major metabolite of tyramine was p-hydroxyphenylacetic acid, the expected end product of monoamine oxidase plus aldehyde oxidase action. This has now been confirmed in rats and mice. There is no evidence that any enzyme other than monoamine oxidase actually degrades the tyramine molecule, although there does appear to be a small degree of conjugation. Since p-hydroxyphenylacetic acid also appears partly in conjugated form, assays for inhibition were done on chromatograms of hydrolyzed urine. These showed only two spots corresponding to tyramine and p-hydroxyphenylacetic acid. Isotope dilution studies† of the combined urine of 5 rats injected subcutaneously with 0.5 μg C^{14} tyramine per g of body weight showed that 88% of the total radioactivity in the urine was present as p-hydroxyphenylacetic acid (mainly free) and 9% as tyramine (about half in free form). In the combined urine of 6 mice injected subcutaneously with 1 μg C^{14} tyramine per g of body weight, of the total radioactivity, 97% was present as p-hydroxyphenylacetic acid (mainly conjugated) and 12% tyramine (mainly free). Within the limits of error of the isotope dilution methods used, these two compounds can account for all the injected radioactivity. In another experiment the expired carbon dioxide of a mouse was found to contain, after 4 hr collection, 2% of the injected C^{14}.

Effect of various substances on monoamine oxidase using tyramine as a substrate. As a method for evaluating monoamine oxidase inhibitors *in vivo,* C^{14} tyramine was administered to mice pre-treated with the test substance and the amount of unmetabolized tyramine in the urine used as a measure of the degree of inhibition. This procedure appears to be valid since the hydrolyzed urine of mice which have been injected with C^{14} tyramine, contains in radioactive form only unmetabolized tyramine and the end-product of monoamine oxidase activity, p-hydroxyphenylacetic acid.

Mice were injected subcutaneously with the inhibitor and after 30 min. injected subcutaneously with 1 μg C^{14} tyramine per g of body weight. Urine was collected from each mouse at 30 min. intervals during a 4-hr period and immediately frozen. When the collection was complete, an approximately equal volume of 6 N hydrochloric acid was added to each urine and hydrolysis effected by heating for one hr in a steam bath. The hydrolyzed urine samples were evaporated to dryness from the frozen state, dissolved in a small amount of water, and paper chromatograms prepared using butanol 80 parts, ethanol 10 parts and ammonium hydroxide 30 parts. This system makes a clean separation of tyramine, Rf 0.9 and p-hydroxyphenylacetic acid, Rf 0.4. Only these two peaks were counted and corrected for background only; the rest of the paper contained negligible radioactivity. Per cent tyramine in the hydrolyzed urine was determined by dividing the c.p.m. in the tyramine peak by the sum of the c.p.m. in both peaks. This method is not intended to give an accurate determination of the absolute value of the tyramine content of the urine. It is designed only to give a base value, approximating the true value, from which deviations caused by inhibitors can be readily observed.

The test substances were chosen because of possible effect on monoamine oxidase. A brief description of each follows: *Ephedrine* inhibits monoamine oxidase *in vitro* using tyramine(7,8) or epinephrine(9) as substrates. It has powerful sympathomimetic properties and potentiates the action of epinephrine. It has been proposed(3) that ephedrine, and possibly other sympathomimetic amines, produce this effect by inhibiting monoamine oxidase and consequently prolonging the action of epinephrine. However, it is also possible that the action is directly on the same receptors acted upon by epinephrine. *Benzedrine and Paredrinol* are also potent sympathomimetic amines and *in vitro* inhibitors of monoamine oxidase(10,9). *Choline p-tolyl*

† In the isotope dilution studies urine was collected at 30 min. intervals for 4 hr.. Carrier tyramine and p-hydroxyphenylacetic acid were added and separation effected by extracting the latter from acidic solution with ether. Both compounds were then recrystallized, using Norite each time, until constant activity was obtained. Tyramine was determined as the picrate; p-hydroxyphenylacetic acid as the free acid.

ether bromide has no obvious sympathomimetic effects. It is reported to be a much more potent *in vitro* monoamine oxidase inhibitor than benzedrine or ephedrine(11). It produces maximum potentiation of responses to epinephrine and norepinephrine in the cat at a level of 10 mg/kg.‡ *Marsilid* (1-isonicotinyl-2-isopropylhydrazine) has only mild sympathomimetic effects. It inhibits monoamine oxidase *in vitro*(12,13) and *in vivo* when tested using epinephrine as substrate (1). *Percaine* is a substitute for cocaine which is a monoamine oxidase inhibitor *in vitro*(8, 14). *Aramine* [levo-1 (m-hydroxyphenyl)-2-amino-1-propanol] is a potent pressor agent; Dapanone (3.4-dihydroxy-α-isopropylaminopropiophenone) is a potent bronchiodilator. They were generously supplied by Dr. Karl H. Beyer of Sharp and Dohme, Inc. *Thyroxin* has been reported to have some relationship to monoamine oxidase activity(15).

The concentrations used were sufficient to produce pronounced pharmacological effects. In some cases (Percaine, Paredrinol) the toxicity imposed a limit on the dose.

Results are shown in Table I.

TABLE I. Effect of Various Compounds on Monoamine Oxidase Activity; C^{14} Tyramine Used as Substrate. All inhibitors injected subcutaneously; after 30 min. C^{14} tyramine injected subcutaneously at a level of 1.0 μg per g of body wt.

Test compound	Conc. of test compound, μg/g	% tyramine in urine
None		15
		16
		18
		22
Ephedrine	50	20
	100	21
Benzedrine (Amphetamine)	20	23
	40	22
Choline p-tolyl ether bromide	10	26
	25	28
	100	32
	100	33
	200	42
Marsilid phosphate	10	27
	25	32
	50	32
	100	31
	150	45
	300	47
	450	47
Percaine (cocaine substitute)	20	20
Aramine	50	14
Dapanone	50	19
Paredrinol sulfate (Veritol, p-hydroxy N-methyl benzedrine)	10	13
"	20	12
1-epinephrine (1 dose 30 min. before, 1 dose 30 min. after tyramine)	.5	19
Thyroxin (doses twice daily for 3 days and once on 4th day 1 hr before tyramine)	1.0	22

Discussion. The data of Table I show that of all the substances tested, only Marsilid and choline p-tolyl ether are effective in inhibiting monoamine oxidase in the intact mouse. Other choline aryl ethers may be inhibitory but only the one compound of this class was examined in the present study.

Several of the compounds which inhibit monoamine oxidase *in vitro* were found to have no effect *in vivo*. This could be due to failure to reach the site of the enzyme because of rapid destruction or for reasons of permeability. It is also possible that *in vitro* preparations of monoamine oxidase have undergone some alteration of the active portion of the enzyme so that response to various substances differs from that in the body.

The powerful sympathomimetic amines, ephedrine, benzedrine and paredrinol showed no inhibitory effect. This strongly suggests that an explanation of their pharmacological activity must involve a mechanism unrelated to monoamine oxidase inhibition.

It is evident from Table I that none of the substances tested is capable of inhibiting monoamine oxidase to a very marked degree when tyramine is used as a substrate.

Summary. 1. Tyramine, labeled with C^{14} in the α-position of the side chain, has been synthesized for use as a substrate in studies of the *in vivo* inhibition of monoamine oxidase. 2. With the exception of a small degree of conjugation, no metabolic route for tyramine could be detected in intact rats and mice other than that due to monoamine oxidase action. 3. A technic for evaluating *in vivo* inhibitory effect on monoamine oxidase has been devised and a number of substances

‡ Personal communication from Dr. Peter Hey, Department of Pharmacology, University of Leeds.

tested. Only 2 active compounds were found; the common sympathomimetic amines were inactive.

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Protection Afforded by Aqueous-Alcohol Solutions of Cortisone Against Dextran.* (20542)

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Attention has been called to the protection afforded by large daily doses of cortisone against the lethal anaphylactoid response of 24-hour adrenalectomized rats to intravenous infusions of the polysaccharide dextran(1). The animals were unequivocally shielded from shock but the procedure employed was slow and expensive since it required a total dosage of 60 mg of a microcrystalline suspension of cortisone (Merck's Cortone), administered over 5 days as a fore-treatment before giving the shock dose of dextran. It was considered desirable to devise a method whereby the dosage could be drastically reduced and the time necessary for adequate prophylaxis lessened without diminishing the effectiveness of the protection afforded. The following experiments demonstrate that, by using a dilute alcoholic solution of cortisone instead of the slowly absorbed microcrystalline suspension, the effective dose of the steroid which will give protection to more than 90% of animals can be reduced from 60 mg to 2 mg or less, and the time required for adequate fore-treatment shortened from 5 days to 6 hours. The simplicity of this modified procedure led the writers to adopt it as a test for screening compounds assumed to have cortisone-like activity.

Materials and methods. 138 virgin female rats averaging 133 g in weight were employed; when ready for use they were adrenalectomized and 24 hours later infused with dextran (Commercial Solvents Corp.; a sterile 6% solution in 0.9% saline); this material is used as a plasma expander for treatment of shock. The dextran was administered intravenously as a single injection, representing 7.5 mg/100 GBW (g of body weight), at the rate of one cc/minute. Rats with intact adrenals tolerate large doses of dextran by vein; however, the 24-hour adrenalectomized animal is extremely sensitive to the material and generally dies within 30 to 60 minutes after infusion(1). If the dextran is given intraperitoneally to intact rats in relatively huge doses, marked edema and erythema may appear(2,3). The striking

* A part of the expenses of this investigation was defrayed by a grant from Sharp and Dohme, Inc., West Point, Pa.

TABLE I. Protection Afforded by Aqueous-Alcohol Solutions of Cortisone against Dextran.

	No. of rats	Avg wt	Dosage, mg/rat	Survival No.	Survival %	No. cyanotic	No. edematous	No. erythematous	No. edematous and cyanotic	No. edematous and erythematous	No. symptom-free
A. Adrenalectomized—controls											
	25	137	—	0	0	25	0	0	0	0	0
B. Adrenalectomized—experimentals											
1	34	129	10*	33	97	15	7	1	3	1	7
2	26	134	5†	25	96	14	1	4	2	4	1
3	29	124	2†	27	93	6	1	8	2	11	1
4	14	125	1†	10	71	10	0	1	2	1	0
5	10	125	0.5†	3	30	7	1	0	1	0	1

* 4 inj. (i.m.) over a 6 hr period prior to dextran infusion.
† 1 " " 6 hr prior to dextran infusion.

anaphylactogenic response of the intact rat to dextran seems to be species specific since other non-adrenalectomized mammalian types, *e.g.*, man or the dog, only occasionally exhibit mild reactions even when infused with large amounts. The free alcohol of cortisone was rendered suitable for parenteral injection by dissolving in 95% warm ethanol and then diluting dropwise with warm distilled water until the clear solution showed a faint haze. 10 mg/cc in 28-29% alcohol can be dissolved in this way. Smaller amounts such as 5, 2, 1 or 0.5 mg/cc were diluted to 10% ethanol or less. One cc was the maximum quantity of fluid given at an injection and the intramuscular route was used exclusively.

Results. The essential data obtained from the study of the soluble cortisone are shown in Table I. The controls (Series A) consisted of 25 rats adrenalectomized 24 hours previously and receiving no salt in the drinking water or prophylactic treatment. All animals succumbed when infused with 7.5 mg dextran per 100 GBW usually within the first hour following infusion. They exhibited profound cyanosis and weakness.

Thirty-three of 34 adrenalectomized rats (B-1) survived after receiving a total of 10 mg cortisone per cc of 28-29% alcohol given in divided doses over a 6-hour period previous to dextran infusion. Although symptoms, such as cyanosis, edema, and erythema, appeared, they were mild and disappeared within a very short time. Since 10 mg of cortisone in solution, administered over a 6-hour period had proven as efficacious in protecting the dextran-infused rats as had 60 mg of the microcrystalline suspension, given as a foretreatment for 5 days, it was considered worthwhile to study the effects of smaller amounts given as a single injection 6 hours before infusing the dextran.

Table I (Series B-2-5) summarizes data from experiments involving lower dosage. Twenty-six animals (B-2) received a single injection of 5 mg of cortisone in 1 cc of 10% ethanol 6 hours before infusion. Twenty-five animals survived; only mild symptoms appeared and were of brief duration. These positive results with lower dosage and but one injection led to further experimentation in

which 2 mg/cc of 10% ethanol were used for prophylaxis (B-3). Of 29 rats so treated, 27 or 93% survived in excellent condition; as in the earlier experiments, the animals displayed only evanescent signs of distress upon infusion. Another series of 14 animals was given a prophylactic dose of one mg cortisone in one cc of 10% alcohol 6 hours before administering dextran; 10 or 71% survived (B-4). A final test was made on 10 adrenalectomized rats (B-5) in which the cortisone dosage was reduced to 0.5 mg/cc in 10% alcohol and given in one injection 6 hours before the infusion. Three rats or 30% lived and these showed severe cyanosis and prostration. The low incidence of protection afforded by this dose of the steroid indicated that the *minimum* effective dose adequate for protection was one mg since this amount protected 71% of the rats.

Discussion. The data in Table I reveal the marked superiority of soluble cortisone as a protective agent over the microcrystalline suspension of the steroid now commercially available and also emphasize the waste of valuable material which evidently occurs when crystalline suspensions are used. Thus by employing a weak alcoholic solution, the effective dose required to insure adequate potection for at least 90% of the rats against dextran was approximately 30 times less than that previously found necessary with the crystalline suspension of cortisone acetate(1). The time required for prophylactic fore-treatment was reduced from 5 days to 6 hours. The best results are obtained when 5-6 hours are allowed to elapse between cortisone injection and dextran infusion. The reason for this is not clear; perhaps this interval represents the optimum time needed for cortisone to suppress or modify the action of immunological mechanisms involved in the lethal response to dextran. The deleterious action of this polysaccharide in the 24-hour adrenalectomized rat is apparently anaphylactoid in nature and essentially similar to that induced by the injection of globin(1) and egg white(4-6). The serological activity of dextrans has been commented upon(7,8). The protective action of small amounts of soluble cortisone upon the dextran-infused 24-hour adrenalectomized rat has been utilized by the writers as the basis for a simple screening test for compounds assumed to have cortisone-like action. Substances possessing small amounts of such activity afford some degree of protection.

Summary. Intramuscular injections of a soluble cortisone are much superior to the microcrystalline suspensions commercially available in affording protection to the 24-hour adrenalectomized rat infused with dextran. The amount of the soluble steroid required to give adequate protection is 30 times less than that found necessary when the suspension is used and the requisite time for prophylactic fore-treatment is reduced from 5 days to 6 hours. The procedure employed in these experiments can be utilized as the basis for a simple screening test for compounds with cortisone-like activity.

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Influence of Severe Hemorrhagic Anemia During Pregnancy on Development of the Offspring in the Rat.* (20543)

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Several extrinsic agents are now known to cause maldevelopment in the mammalian embryo if properly applied during the gestation period. These include: maternal dietary deficiency of vit. A(1,2,3), riboflavin(4,5), pantothenic acid(6), folic acid(7,8), and possibly vit. B_{12}(9); at least 2 viral diseases, namely, German measles(10) and hog cholera(11); injections of such chemicals and drugs as nitrogen mustard(12), trypan blue(13) and cortisone(14); such additional maternal conditions as hypoxia(15) and hypervitaminosis A(16); and finally, exposure to ionizing radiations(17,18). In no case is the mechanism of action on the embryo known, but all of these agents with the possible exception of the last must act either on or through the maternal organism. Several of them are known to cause more or less drastic physiologic alterations in the mother, and the question arises as to what extent this maternal reaction may affect the embryo. As part of a series of studies on this subject, the following investigation of hemorrhagic anemia during pregnancy in the rat was undertaken.

Methods. Anemia was induced by allowing free bleeding from the freshly severed tail on each of 3 successive days. By snipping off a segment of the tail from an animal lightly anesthetized with ether, it was usually possible to collect 4 to 6 cc of blood within a few minutes. The procedure was repeated on the 2 succeeding days, with the result that frequently total quantities equivalent to 6, 7 or more cc/100 g body weight were withdrawn over the 3-day period. Each time blood was removed a 5-drop sample was heparinized for hematocrit reading, and for the same purpose a few drops were taken the day after the last bleeding and at irregular intervals thereafter by opening a caudal vessel. The periods of bleeding were begun at various times during pregnancy so that the height of maternal anemia would coincide with known stages in embryonic development. Accordingly 31 pregnant females were bled on the 7th, 8th and 9th days to cause severe anemia on the 9th day when the mesoderm is forming and on the 10th day when organogenesis is beginning. Forty females were bled on the 9th, 10th and 11th days and 37 on the 11th, 12th and 13th days to produce anemia during the period of most active organogenesis between the 11th and 14th days of gestation. Twenty-five females were bled on the 13th, 14th and 15th days so that severe anemia would occur during the period of late organogenesis and early histogenesis on the 15th and 16th days. Pregnancy was dated from 9 AM of the morning on which sperm were found in the vaginal smear, the embryos being considered *one day of age* 24 hr later. To verify pregnancy and count the number of embryos the females were laparotomized immediately following the first bleeding. Mothers surviving the 3 bleedings were allowed to live until the 20th day of gestation when they were killed and the young again counted, then removed, weighed, examined under a magnifying lens for malformations, and fixed. Two young from each litter were fixed in 95% alcohol for clearing by the Schultz-Dawson method in order to evaluate skeletal development. The remainder were fixed in Bouin's fluid and later dissected or sectioned.

Results. The removal of total quantities of blood equivalent to 5 cc or more/100 g body weight caused a precipitous drop in hematocrit values (Table I). In general the extent of drop was proportional to the total quantity of blood removed, but this was subject to considerable variation. The effects of anemia on survival of the mother and her offspring were more closely correlated with quantity of blood removed than with hematocrit reading, for which reason the following

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TABLE I. Hematocrit Reading before and after Induced Hemorrhage on 3 Successive Days, Beginning at Different Times in Pregnancy.

Days bled	Avg hematocrit readings (%)			
	Before first bleeding	24 hr after 3rd bleeding (amt removed—cc/100 g body wt)		
		5–5.9 cc	6–6.9 cc	7 or more cc
7, 8 and 9	43.3 ± 2.8	21.0 ± 3.2	15.8 ± 1.3	16.0 ± 2.6
9, 10 and 11	42.2 ± 2.9	19.3 ± 1.7	17.4 ± 2.3	14.9 ± 2.1
11, 12 and 13	39.2 ± 4.9	18.1 ± 1.7	16.5 ± 2.8	14.8 ± 1.9
13, 14 and 15	39.7 ± 2.6	17.8 ± 3.0	16.6 ± 1.8	14.4 ± 1.4

± = Stand. dev.

TABLE II. Effects on Gestation and on Offspring of Removing Various Quantities of Blood from Female Rats at Different Times in Pregnancy.

Total blood withdrawn (cc/100 g body wt)	No. of mothers	Pregnancies terminating before 20th day		Pregnancies going to 20th day			
		% maternal death	% complete resorption	%	% young resorbed*	Avg wt young (g) †	Condition of young
Bled on 7, 8 and 9th days pregnancy							
4–4.9	7	0	0	100	9	3.57	All normal
5–5.9	9	0	33	67	7	3.56	"
6–6.9	7	43	57	0	—	—	—
7–	8	62	13	25	4	3.38	All normal
Bled on 9, 10 and 11th days pregnancy							
5–5.9	12	8	25	67	6	3.39	1 litter retarded
6–6.9	15	20	27	53	18	3.20	3 young malformed; 1 litter retarded
7–	13	23	38	39	12	3.18	"
Bled on 11, 12 and 13th days pregnancy							
5–5.9	7	0	0	100	13	3.41	All normal
6–6.9	21	38	0	62	10	3.38	"
7–	9	44	0	56	5	3.11	1 litter retarded
Bled on 13, 14 and 15th days pregnancy							
5–5.9	5	20	0	80	11	3.35	All normal
6–6.9	13	54	0	46	0	3.30	"
7–	7	71	0	29	0	3.10	1 litter retarded

* May reach 14% in litters from non-anemic mothers.
† Mean wt of young from non-anemic mothers = 3.34 ± .34 g.

data are presented in relation to quantity of blood removed. Ten non-pregnant adult females bled amounts equivalent to 6 cc or more/100 g body weight showed lowering of hematocrits comparable to those in Table I. Six died within 2 days following the last bleeding, indicating that the treatment was as deleterious to non-pregnant as to pregnant females.

Bleeding on 7-9th days. Removal of less than 5 cc of blood/100 g body weight was without effect on the duration or outcome of pregnancy (Table II). All mothers survived and continued their pregnancies to the 20th day. Nine per cent of offspring underwent intrauterine resorption after laparotomy on the 7th day, but this is within normal range, which may reach 14% in the offspring of non-anemic, laparotomized females. The mean weight of surviving young was normal, and no developmental defects were observed in dissected, sectioned or cleared specimens. Removal of 5 to 5.9 cc of blood/100 g body weight also did not kill any mothers but did

result in early termination of pregnancy in one-third of the cases due to resorption of the entire products of conception (Table II). In pregnancies which continued to the 20th day, however, the offspring were normal in all respects. Hemorrhage in excess of 6 cc/100 g body weight caused early termination of pregnancy in all but 2 of 15 cases, due either to maternal death or complete resorption of young. It is particularly noteworthy that the 2 pregnancies which survived this drastic treatment yielded litters of normal offspring.

Bleeding on 9-11th days. Again removal of quantities of blood between 5 and 5.9 cc/100 g body weight caused premature termination of approximately one-third of pregnancies, but those litters which reached the 20th day were essentially normal (Table II). Young in one of 8 surviving litters were retarded in growth or skeletal development but none was malformed. When 6 to 6.9 cc of blood/100 g body weight were removed, a more pronounced effect was observed in a few cases. Although 8 of 15 pregnancies continued to the 20th day, the mean rate of prenatal death in the surviving litters was increased, one litter contained young that were retarded in growth, and 2 others contained a total of 3 grossly malformed young (Table II). No one of these deviations is in itself convincing evidence that maternal anemia interfered with development, but in the aggregate they indicate that anemia of sufficient severity at this time (11th day and subsequently) has some tendency to affect the offspring. That this tendency is slight and variable is demonstrated by results obtained from mothers bled 7 or more cc/100 g body weight. Although only 5 of 13 such pregnancies continued to the 20th day, there was no significant abnormality in the young. When surviving young from all mothers bled in excess of 6 cc/100 g body weight are combined, it is found that only 3 of 117 or 2.6% were malformed. Thus, it is concluded that although an occasional abnormality may be produced at this time, maternal anemia usually causes either early termination of pregnancy or else permits continuation of pregnancy without detriment to the young. The 3 malformed young showed various skeletal dysplasias and distortions, such as shortening of the snout and extremities. Two exhibited combined polydactyly and syndactyly. The eyes were malformed in 2 instances, the optic cup in one and the lens in another. Aside from developmental defects, all were noted to be edematous at delivery and on sectioning to have foci of degeneration in the central nervous system. Basal ganglia, certain regions of cerebral cortex, and the anterior horns of the spinal cord were the commonest sites of degeneration, and often in these regions signs of localized hemorrhage were evident. Such a pattern of abnormality has not been encountered in several hundred young rats from non-anemic mothers examined in this and other experiments. This, together with the fact that all 3 animals were similarly affected, suggests that the abnormalities were a consequence, however rare, of the maternal anemia.

Bleeding on 11-13th days and on 13-15th days. The effects of hemorrhage during these 2 periods were essentially similar except that bleeding in the later period caused a higher rate of maternal death (Table II). In neither instance was there a significant effect on the young in pregnancies continuing to the 20th day, although one litter from each group of mothers bled more than 7 cc/100 g body weight contained young moderately retarded in growth. Unlike some bled at earlier periods none of these mothers was recorded as having resorbed her entire litter. There was, nevertheless, evidence that death of all the fetuses sometimes preceded that of the mother. Several of the mothers were autopsied soon after spontaneous death, between the 14th and 16th day of gestation; and in at least 4 of these the fetuses were found to have been in process of resorption at the time of maternal death. Whether the fetal death contributed to the maternal death in such cases is uncertain, but it is noteworthy that at the time the mothers died the fetuses, not including membranes and decidua, each weighed 0.2 to 0.5 g. The resorption of several g of necrotic fetal and accessory tissue certainly placed an added burden on an already impaired maternal organism. Maternal death occasionally attributable to death and resorption of the fetuses

seems likely.

Comment. These results indicated an all-or-none mechanism concerning the welfare of the embryo under conditions of maternal hemorrhagic anemia. Either the anemia had little or no effect on the offspring, or its effects were such as to cause maternal death or complete resorption of the conceptus. There was virtually no intermediate condition in which the offspring survived, but under duress or deprivation sufficient to cause maldevelopment. Evidently the physiologic stresses resulting from hemorrhagic anemia are of a nature that affect the viability of the mother or interfere with continuation of her pregnancy before they affect the processes of development. Only bleeding of the mothers on the 9th, 10th and 11th days showed any tendency to interfere with development in surviving embryos, and this was so limited that hemorrhagic anemia at this or any other time can hardly be regarded as a real hazard to normal embryonic development in the rat.

This is in contrast to the action of the known teratogenic agents with which it is usually possible to demonstrate three dose-response relationships, that is, three degrees of effect of the agent upon the offspring. The agent in mild or moderate dose may have no effect, or at somewhat higher doses it may prove universally fatal to all embryos or to the mother. Between these limits exists what can be called the teratogenic zone, a more or less narrow zone in which embryonic life is able to continue but frequently at the expense of impairment or aberration in normal development. A significant teratogenic zone was not clearly demonstrated for hemorrhagic anemia in the rat. This implies that the mere placing of severe physiologic stress upon the pregnant mother is not enough to cause maldevelopment among the offspring. To act as a teratogenic agent it is probably necessary that such stress bear a specific relation to the needs or welfare of the embryo at the time the agent is operative.

Summary. Pregnant rats were rendered severely anemic by permitting free bleeding from the freshly severed tail on 3 successive days. This frequently caused early termination of pregnancy as a result of maternal death or resorption of the entire litter, but litters from mothers surviving this treatment were essentially normal. Only when bleeding was begun on the 9th day was there a minimal effect on the offspring, manifested by malformations in 2.6% of the young, a mild degree of retardation of growth in 4 of 13 litters, and a slight increase above the normal rate of intrauterine resorption in litters reaching term. Since even these minimal effects were not observed after hemorrhage at other times during gestation, it is apparent that maternal hemorrhagic anemia represents no particular hazard to the offspring in surviving litters. This suggests that severe physiologic stresses during pregnancy, however severe, are not always capable of causing abnormality in the offspring.

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Effect of Cortisone Administration on Intracellular Composition of Rat Liver.* (20544)

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Profound alteration in the histology of rabbit(1,2) and rat(3) hepatic parenchyma and changes in nucleic acid content of such livers have been reported(1,3,4) following cortisone administration. These changes are characterized by increase in cell size, accumulation of glycogen and marked decrease in cytoplasmic basophilia. Since the major contribution to cellular basophilia (toluidine blue and gallocyanin chromalum staining) is the pentose nucleic acid (PNA) of the particulate material of the liver cell, it seemed important to investigate the effect of cortisone administration upon the PNA content and the total nucleic acid contents of the liver.

Methods. Three groups of Wistar strain rats, 100-200 g in weight, were maintained *ad libitum* on a Rockland rat diet. Group I: Normal controls. Group II: Cortisone-treated for 5 days and sacrificed 24 hours later. Group III: (Recovery group); cortisone-treated for 5 days but not killed until 8 days after the last dose of cortisone. Animals in Groups II and III received 25 mg cortisone† by intramuscular injection daily for 5 days. All animals were killed after a 24-hour fast by exsanguination through the hepatic vein during light ether anesthesia. Control animals were similarly fasted and killed. Livers were removed rapidly, rinsed in isotonic saline and promptly expressed through the fine mesh of an iced tissue press. The tissue was then weighed, suspended in iced, slightly alkaline 0.85% saline, and further homogenized in a glass Potter-Elvehjem grinder. The brei was then diluted with additional alkaline saline so that each ml of solution was equivalent to 0.2 g of whole wet liver. The centrifugal separation was essentially that described by Huseby and Barnum(5,6), with the following fractions being obtained:

Fraction		Relative gravitational force	Time for sedimenting (min.)
Nuclear	(N)	1400	4
Mitochondrial	(Mit)	24000	5
Microsomal	(Mi)	24000	90
Ultracentrifugal	(U)	110000	90
Nonsedimentable	(S)		

The crude fractions were treated as outlined by Barnum and Huseby(7); chemical separation of the phosphorus-containing compounds was carried out essentially as described by Schneider(8), and subsequent analyses for pentose, desoxypentose, phosphorus, nitrogen, glucose and dry weight performed as previously described(1). Histochemical examination of sections was conducted as outlined by Williams, Lowe, and Thomas(2).

In each instance, the phosphorus as well as the carbohydrate content of every particulate nucleic acid fraction was determined in duplicate. The theoretical carbohydrate content based on phosphorus was calculated, assuming a statistical tetranucleotide residue with a pentose-to-phosphorus ratio of 4.84 for pentose nucleic acid(5). If the theoretical and actual values were not within 5% of each other, the results were discarded, except in the case of nuclear PNA, when discrepancies tended to be as great as 10-15%.

Results. Table I‡ summarizes the data ob-

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† Cortisone acetate in a water suspension containing also 0.9% benzyl alcohol, 0.9% sodium chloride, 0.4% polyoxyethylene sorbitan monooleate and 0.5% sodium carboxymethylcellulose was used. This was generously supplied by Dr. Elmer Alpert of Merck and Co., Rahway, N. J.

‡ While the text uses the notation PNA and DNA, all tables indicate values for ribose and desoxyribose, since these were the substances actually measured.

TABLE I. Effect of Cortisone Administration upon Rat Liver Composition.*

Group	Type of exp.	μg/g wet liver — Rib†	DRib‡	Avg Rib/DRib	mg/g wet liver — Glucose	Nitrogen	% dry wt
I	Normal	4409 ± 75 (21)	955 ± 31 (21)	4.70 ± .01	5.21 (5)	35.3 ± .04 (6)	28.8 ± .01 (5)
II	Cortisone	3449 ± 87 (15)	751 ± 15 (15)	4.61 ± .13	56 ± 5.8 (13)	32.1 ± .7 (9)	30.5 ± .03 (10)
III	Recovery§	4786 ± 226 (5)	930 ± 31.7 (5)	4.98 ± .22 (5)	5.8 ± .71 (5)	36.2 ± .11 (5)	30.2 (2)

* Avg and stand. error of mean.
† Ribose × 2.
‡ Desoxyribose.
§ Animals killed 8 days after last dose of cortisone.
Figures in parentheses refer to No. of exp.

TABLE II. Effect of Cortisone Administration upon Composition of Rat Liver Cell Cytoplasm.*

Group	Type of exp.	Rib† — Mit‡	Mi§	U‖	S¶
I	Normal	158 ± 14 (7)	1029 ± 35 (8)	235 ± 21 (5)	197 ± 9 (7)
II	Cortisone	0 (15)	0 (15)	1003 ± 56 (13)	276 ± 10 (17)
III	Recovery**	392 ± 43 (4)	1168 ± 159 (5)	229 ± 28 (5)	296 ± 3 (5)

* Avg and stand. error of mean. † Ribose × 2, μg/g wet liver. ‡ Mitochondria. § Microsomes. ‖ Ultracentrifugable. ¶ Non-sedimentable. ** Animals killed 8 days after last dose of cortisone.
Figures in parentheses refer to No. of exp.

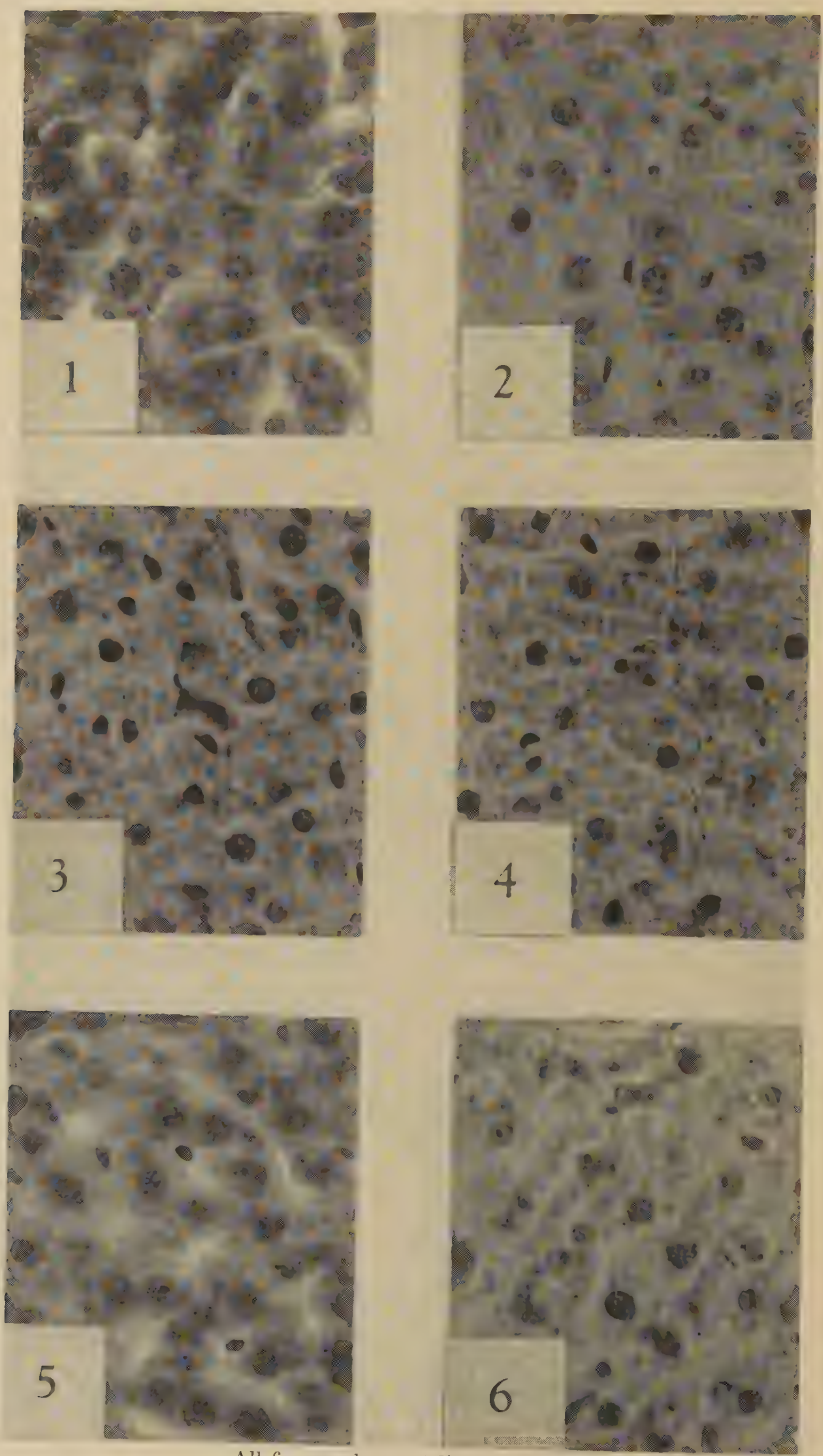
tained on the effects of cortisone upon the composition of rat liver. Following cortisone administration for 5 days (Group II) the PNA and DNA per g of liver decreased as compared to controls (Group I), while the ratio PNA/DNA remained essentially unchanged. Hepatic glucose content increased 10-fold while the nitrogen and water content were quite similar to that found in the normal animals. Eight days after cortisone administration (Group III), the liver composition had returned to that of untreated controls.

Table II shows the results of cytoplasmic fractionation. Mitochondria and microsomes from normal liver were rich in PNA. The most striking finding following cortisone administration (Group II) was the inability to demonstrate mitochondrial and microsomal PNA, although in each instance a small pellet was obtained by centrifugation. The figure for mitochondria of Group II given in Table II is zero, although in 2 out of 15 experiments significant material was obtained. Appreciable PNA was obtained in the microsomal fraction on only one occasion. The U fraction increased almost 5-fold, while the nonsedimentable nucleic acid increased about 30%. While the possibility exists that the increase in the U fraction represented mitochondria and microsomes that sedimented under increased G, this possibility was discarded because of two considerations. Studies (unpublished) using P_{32} indicate the normal U relative specific activity (RSA) to be 4 times that of mitochondria or microsomes. The RSA of U in livers of cortisone-treated animals is es-

TABLE III. Effect of Cortisone upon Composition of Rat Liver Cell Nuclei.*

Group	Type of exp.	DRib†	Rib‡	Avg Rib/DRib
I	Normal	955 (17)	57.1 ± 2.6 (7)	.06
II	Cortisone	751 (15)	43.7 (4)	.058

* Avg and stand. error of mean.
† Whole liver desoxyribose, μg/g wet liver.
‡ Nuclear ribose × 2, μg/g wet liver.
Figures in parentheses refer to No. of exp.



All figures show rat liver, ×750.

Fig. 1-4, sections stained with aqueous toluidine blue.

FIG. 1. Normal rat, buffer (pH 6.8) control section. Abundant basophilic granulation in cytoplasm.

FIG. 2. Section of same liver placed in ribonuclease-buffer solution (pH 6.8) for 5 hr. Basophilia greatly reduced by enzymic hydrolysis.

FIG. 3. Cortisone-treated rat, buffer-control section. Very small amount of cytoplasmic basophilia.

FIG. 4. Same liver as in Fig. 3, but section was subjected to ribonuclease. No significant reduction of basophilia by action of enzyme.

Fig. 5 and 6, sections stained by gallocyanin-chromalum at pH 2.5.

FIG. 5. Normal rat. Large amount of cytoplasmic basophilia.

FIG. 6. Cortisone-treated animal. Very sparse cytoplasmic basophilia.

sentially the same as that of the normals. Dilution of an active fraction with an inactive one should produce significant decrease in RSA of the active one. This was not observed. The second consideration arises from microscopic examination of films made of the U fraction using osmic acid fixation. No significant mitochondrial material was seen.§ Table II further shows that in the "recovery" animals (Group III) a return to almost normal values was noted except for what appears to be over production of Mit and S PNA.

Table III summarizes the studies of nuclei. Following cortisone treatment (Group II), nuclear PNA was reduced on a per g basis as compared to control animals (Group I), but the amount of PNA per unit of DNA was almost identical in the two groups.

The sum of isolated cytoplasmic and nuclear PNA does not equal the amount found in the total liver brei in either the control or experimental animals. This is probably due to the fact that wash waters were not analyzed. However, the loss of PNA was of the same order of magnitude (approximately 50%) in each group of animals.

The histologic findings are illustrated in Fig. 1-6. In contrast to normal animals, following administration of cortisone for 5 days, essentially no cytoplasmic basophilia could be demonstrated by staining with aqueous solutions of toluidine blue or with gallocyanin-chromalum at pH 2.5. The small amount of residual basophilia was not significantly decreased by ribonuclease hydrolysis. While the demonstration of cytoplasmic basophilia by these methods is considered to be specific for PNA(2,9), such methods are not specific for mitochondria. However, it is generally believed that the major contributor to cytoplasmic basophilia of liver cells is the PNA normally associated with mitochondria and microsomes. In no sections was there evidence of necrosis.

Eight days after cessation of injection of cortisone, cytoplasmic basophilia and acidophilia had increased, and returned to levels observed in normal rats.

Discussion. The findings demonstrate that no mitochondrial or microsomal PNA is isolated by centrifugation from livers of rats that had previously received cortisone, when isotonic alkaline saline is used as the suspending medium. Furthermore, the hepatic cells are altered by this hormone to the extent that the cytoplasm no longer responds characteristically to staining methods that demonstrate PNA. From these results, however, it cannot be concluded that mitochondria or microsomes as organized particulate units are not present in the livers of cortisone-treated rats.

Since the ratio of total PNA to DNA in the liver was not significantly altered by cortisone, one may conclude that on the basis of considerations previously presented(1) there was probably no overall loss of PNA; however, the location of PNA in the cytoplasm seemed altered. Since the particles making up the U fraction are not visible in the light microscope, an increase in PNA in that fraction should not affect cellular basophilia. The increase of PNA in U and S was approximately equal to the amount normally present in mitochondria and microsomes. These two findings suggest that one effect of cortisone is to cause a translocation of PNA in liver cells from mitochondria and microsomes to U and S. If mitochondria and microsomes are not removed from liver cells by cortisone, both these particulates are nevertheless grossly altered, for they no longer seem to be associated with significant amounts of PNA.

§ We are indebted to Dr. Daljit Sarkaria for the preparation and interpretation of these films.

It is doubtful that all mitochondria are removed from liver by cortisone, since these particles are the locus of several enzymes, without whose function metabolism seems unlikely(10). The observation of alterations in mitochondrial material in liver cells was an unexpected finding in these experiments. However, several carcinogenic dyes have been reported to change not only the PNA content of mitochondria but also the number per liver cell(11,12). Accompanying these changes there was an alteration in the amount of succinoxidase activity per cell(13,14).

The essentiality of microsomes and microsomal PNA for cellular metabolism is not definite. It is interesting in this connection that Barnum and Huseby(15) have recently shown that PNA can be removed *in vitro* from microsomes of mouse mammary gland infected by the milk agent virus without any loss of infectivity of such microsomes.

Elucidation of the problem of whether or not mitochondria and microsomes are truly removed from liver cells by cortisone may be accomplished by studies using staining methods specific for mitochondria and electron microscope examination for microsomes.

Summary and conclusions. (1) PNA was not demonstrated in the mitochondria or microsomes obtained by differential centrifugation from liver cells of cortisone-treated rats. (2) The characteristic hepatic parenchymal basophilia usually demonstrated by staining with toluidine blue and by gallocyanin-chromalum at pH 2.5 was also lacking. (3) Eight days after cessation of cortisone administration, liver composition had returned to normal.

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Calorimetric Estimation of Catalase Activity.*† (20545)

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The estimation of catalase activity is generally carried out by measuring the amount of oxygen evolved or the amount of peroxide decomposed(1-5). Though hydrogen peroxide is generally used, perborate has also been

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used as a substrate(6). It is desirable, whenever possible, to have methods of measurement which involve experimentally independent quantities. The amount of heat liberated may be used to measure activity. The present communication presents a simple and rapid method for the estimation of catalase activity which is particularly convenient for the assay of blood. The method requires the measurement of the maximum temperature rise after the addition of the enzyme. A clinical thermometer is convenient to measure the maximum temperature.

Method. To a plastic test tube, 15 mm in diameter, 9 cm length and weight of about 2 g, add 1 small drop of capryl alcohol and 5 ml of aqueous solution, at 25°C, containing 0.50 ml of Superoxol (30% H_2O_2) and about 1 ml of nearly neutral phosphate buffer (1 part 0.1 M Na_2HPO_4 to 1 part 0.04 M NaH_2PO_4). Place a thermometer into the tube and set the tube into a test tube holder consisting of a block of wood with holes about 8 cm deep and 1.8 cm diameter. The holes should be about 4 cm or more between centers. Each hole should have a collar at the top which centers the tube in the hole and the bottom of the hole should be tapered so that only a small ring shaped area at the bottom of the test tube contacts the wood. If the room temperature is not close enough to 25°, glass test tubes filled with water at 25°C may be kept in the holder for some time in advance and the temperature checked just before use. The wooden block must be kept dry. Just before adding the blood sample, the thermometer is read. The temperature should be 24.5 to 25.5°C. The blood sample, 0.01 ml, is blown out slowly on to the bulb of a clinical thermometer (scale range preferably 33-43°C and shaken to below 33°) which is then lowered into the solution and used to stir for 15 seconds. Should there be difficulty in blowing the blood from the pipette, the tip should be moistened with water. The final temperature may be read at any time after about 8 min. If at this time the clinical thermometer is found not to have changed due to the fact that a temperature of 33°C or above has not been achieved and if not more than 10 min. have elapsed, a fairly satisfactory estimate of the temperature rise can be made by inserting a low heat capacity thermometer, warmed to about 30°, and the temperature read after stirring.

Modifications. The blood may be diluted up to about 0.3 ml with buffer if the initial volume is adjusted and if the temperature of the added solution does not differ from 25° by more than one or 2 degrees. However, the samples are less stable when diluted and should be iced if not used within a few minutes. If whole blood is oxalated and iced, the activity drops very slowly, about 10% in a week. Occasionally it may be convenient to work with diluted samples of blood. In this case, 0.01 ml blood in 4.5 ml buffer solution which has been kept cold is quickly brought to 25° in a plastic tube. A drop of capryl alcohol is then added. The tube is placed into the wooden test-tube holder and its temperature recorded. Then 0.50 ml of Superoxol at 25° ± 1°C is added and the solution is stirred for 15 seconds. The maximum temperature rise is then read any time after about 8 minutes. If the volumes are all doubled the temperature rises will be somewhat more than 1% higher. If the volumes are increased ten fold, using large plastic centrifuge tubes, the temperature rise will be about 5% higher when 0.015 ml or more of an average blood sample is used for each 5 ml final volume. If the volume is cut down to 2 ml, the temperature rise is decreased only by about 5%. When glass test tubes 10 cm long, 13 mm diameter, weight 9 g are used instead of plastic tubes, essentially the same results will be obtained provided that 1 ml of water is left out and the tubes are insulated by placing them into a roll formed by three double facial tissues, each folded in half.

The results are rather insensitive to pH. When only the monobasic phosphate (pH 4.5) is used the activity is only reduced by about 10% whereas when only the dibasic phosphate (pH 8.9) is used, the temperature rise is not significantly affected. The results are also rather insensitive to the amount of buffer added unless considerable more is added than that recommended. On the other hand, leaving out the buffer had no appre-

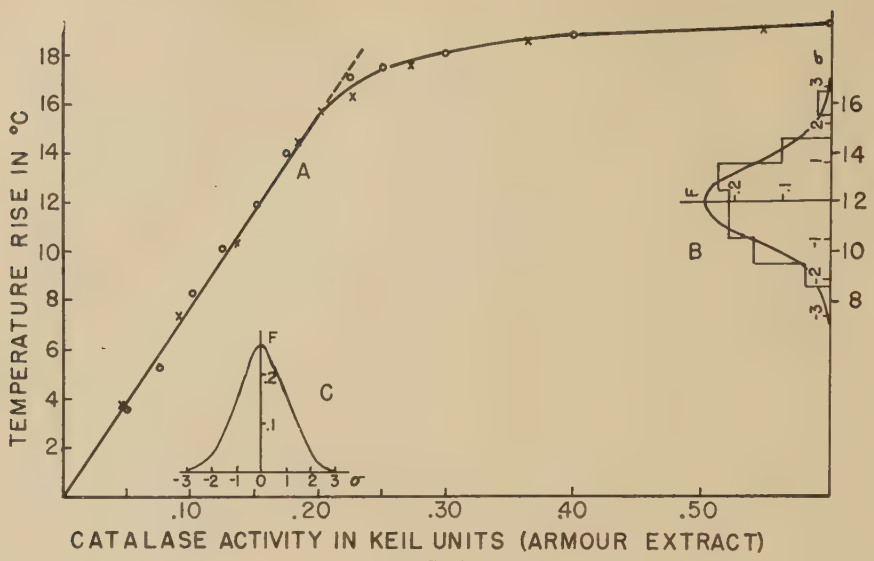

FIG. 1.

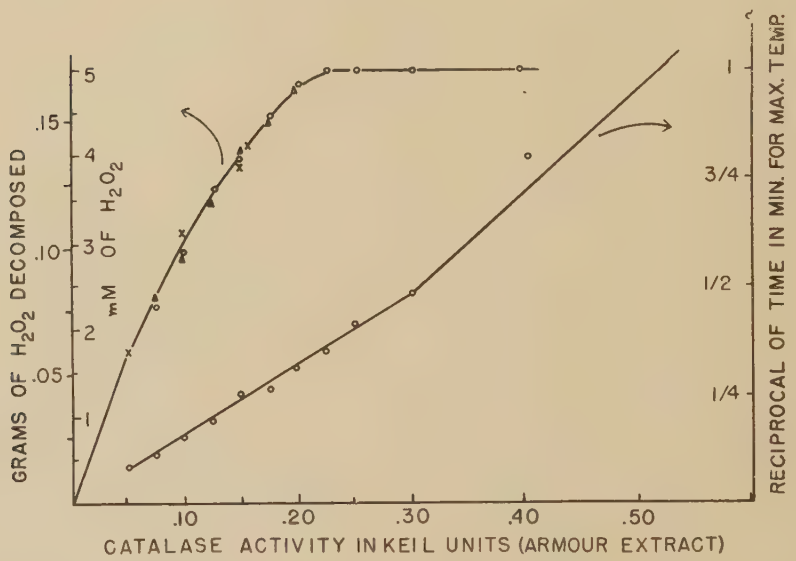

FIG. 2.

ciable effect. If the initial temperature is appreciably greater than 25°C the temperature rise will be more rapid at first but the total rise will be smaller by about 2% for each degree.

Standard curve. In Fig. 1 (curve A) are shown the results of measurements of the temperature rise due to various amounts of Armour's catalase, the activity being verified by the method defining the Keil unit. From the graph it can be seen that the temperature rise is very nearly linear with the amount of enzyme added up to 16°. The results in the graph are in part from the method in which the peroxide is added and in part from the method in which the enzyme was added. The temperature rise is relatively insensitive to the concentration of peroxide if the activity is within the linear range. The curves B and C will be discussed subsequently.

In Fig. 2 are shown the reciprocal of the time for maximum temperature as well as

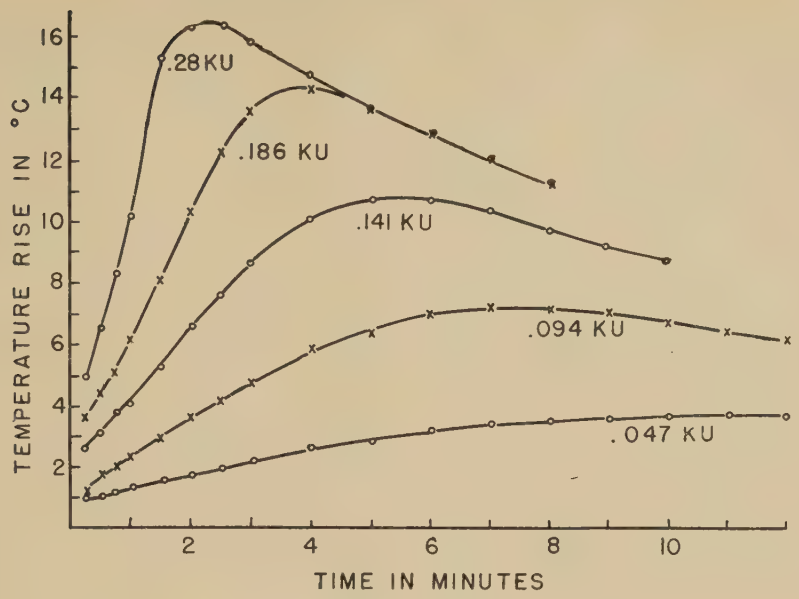

FIG. 3.

the amount of H_2O_2 decomposed after several hours. In this connection it may be pointed out that for enzyme concentrations up to 0.12 Keil units, the amount of peroxide decomposed after a long time (somewhat more than 10 min. for 0.12 Keil units) under the above conditions is the same as that decomposed in 10 min. under the conditions defining the activity unit. The latter value is about 86% of the amount that would be decomposed in an hour, the enzyme being practically all destroyed at that time. Note that one Keil unit decomposes 1 g H_2O_2 in 10 min. at pH 7, 25°C, when the H_2O_2 concentration is at 0.2% and the recommended phosphate-citrate-acetate buffer is used.

Since the heat loss varies somewhat with the materials used for tubes and insulation it may be desirable to give as a reference a standard maximum temperature rise. This is done as follows. At time zero add about 0.01 ml blood to the peroxide solution containing 5.0 mM H_2O_2 (very close to 0.50 ml 30% H_2O_2), starting at 23-24°C, and adding a larger drop (0.04 ml) of capryl alcohol. Stir for about 15 seconds, then add a second 0.01 ml sample at 30 seconds and a third at 60 seconds, stirring just before and after each addition. A maximum temperature rise of about 19°C will be reached at very close to 1½ min. The temperature rise will be roughly linear over this period of time and relatively independent of the activity of the sample used.

The concentration of the peroxide may be verified to within a few per cent by estimating the heat liberated under the conditions for the standard temperature rise. The heat loss may be estimated by warming the tube containing 5 ml of aqueous solution to slightly above 43°C. When the temperature is 43.0°C lower the tube quickly into the wooden block and stir and record the temperature at 45″. At this time, which is half the time to the maximum, the temperature fall should be about 1.5°C. This added to the measured temperature rise is the corrected temperature rise (ca. 20.5°C). The heat capacity of the system is 5.0 for the aqueous solution minus about 0.1 for the volume change, due to the reaction, and 0.25 for the thermometer, 0.3 for the plastic tube and 0.05 for the added solution and capryl alcohol. The product of the heat capacity and the temperature rise ($5.5 \times 20.5 = 113$) should be equal to the heat liberated less about 2 calories to account for the loss due to the specific heat of O_2 and evaporation of water. If we take 23.4 cal/millimol then the heat liberated should be 115 calories.

Time course of heat liberation. The time course of liberation of heat was followed for a few values of the enzyme concentration. In these cases 0.3 ml H_2O_2 was left out in order to compensate for the difference in heat capacity between the thermometer used and a clinical thermometer. The results are shown in Fig. 3.

If the solution is diluted by about 4-fold, the heat liberated becomes very insensitive to the peroxide concentration. Also the heat loss is substantially less. Even with a constant temperature outer wall the heat rise will be nearly 5 degrees. The heat loss now can be quantitatively measured and the instantaneous temperature recorded continuously. If it is desired not only to assay a preparation but also to measure reaction rates, the interpretation of the measurements are less complicated if the temperature is nearly constant. This condition can be met if the solution is further diluted so that the effect of the temperature change on the reaction rate can be neglected.

Comparison of the time course of activity as measured by heat liberation, oxygen evolution and titration of residual H_2O_2. For the purpose of estimating an absolute amount of heat liberated, it is easier to use larger volumes, keeping the surrounding temperature constant. The temperature loss is then smaller and more easily estimated. In order to estimate the amount of O_2 evolved at any time, it is necessary to keep the solution volume small and to shake the solution violently before making readings so as to reduce the effect of the supersaturation by O_2. In the results to be discussed in this section generally 10 ml of solution were placed into a 20 ml Warburg flask connected by means of very small rubber tubing to two 100 cc syringes immersed in an ice bath. The volumes of O_2 liberated were therefore up to 112 cc at 0°C, 760 mm Hg. The flask was insulated so that its temperature would closely parallel that of the corresponding solution used to follow the temperature. The effect of the temperature change of the 10 cc of gas in the flask was slight. The volume of O_2 liberated could then be followed with time measured from the time of mixing of the enzyme and the solution. The values thus obtained could be compared with the amounts of heat developed as well as with the results of titration with $KMnO_4$ of the remaining H_2O_2 for given experimental conditions. Some typical results are shown in Table I. The results in the last two columns are for a case in which the starting temperature was about 4°C higher than the temperature for the others (25°C). The results of the three methods can be seen to be very nearly the same except for a tendency for the heat liberation to be slightly higher than the others. The values given in the table are fractions of their respective maximum possible values.

TABLE I. Comparison of O_2 Liberation, Heat Evolution and Titration of Residual H_2O_2.

Time (min.)	.19 KU			.09 KU		.10 KU	
	Gas	Tit.	Heat	Gas	Tit.	Tit.	Heat
1	.25	.26	.27	.11	.12	—	—
2	.44	.46	.46	.17	.17	.32	.34
3	.67	.67	.71	—	—	—	—
5	.98	.95	.96	.35	.36	.56	.59
10	.99	.99	.99	.58	.58	.59	.61
20	—	—	—	—	.58	.60	.61

Results with human subjects. Blood samples, drawn for routine examinations on 58 nominally normal individuals, were used to test the method. The samples were shaken with oxalate and refrigerated immediately after being drawn. The results of such measurements are shown in Fig. 1, the frequency distribution, F, of the temperature rise being shown on the right (curve B), that of the activity on the lower scale (curve C). In curve B, the scale is the same as that on the left. Also given are the deviates σ of the distribution in terms of the standard deviation, the smooth curve being the normal error curve. For curve C the abscissa is the same as that of curve A. In this case only the normal error curve is given. The standard deviation is that of the average of various groups. The variation between groups was not very large.

A few trials on rabbit and mouse blood suggest that 0.02 ml of rabbit blood in 5 ml final volume is satisfactory whereas for mouse blood 0.02 ml in 2 ml final volume gives a sufficient temperature rise.

Although the method has been used with

blood samples, it may be useful for estimating the activity in tissues. This is especially the case if this method can be used where there is marked interference with the titration of remaining peroxide.

Summary. A method is described for estimating catalase activity by the amount of heat liberated. The latter is estimated from the temperature rise of the solution, the maximum temperature being conveniently recorded by a clinical thermometer. The average temperature rise of the solution to which 0.01 ml of human blood was added was found to be about 12°C, corresponding to a liberation of 0.4 M or 14 g of H_2O_2 by each ml of blood. Some comparisons are made of the time course of the liberation of heat with that of the evolution of O_2 and the disappearance of H_2O_2 as estimated by titration.

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Origin and Development of the Urogenital Union in the Chick.* (20546)

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The literature(1) on the origin of the rete in amniote embryos reveals 3 general viewpoints: (1) that the rete derives from the renal corpuscles of the mesonephros and joins the gonad secondarily; (2) that the rete arises from the germinal epithelium and only secondarily becomes associated with the mesonephros; (3) that the rete differentiates within the mesenchyme between the gonad and the mesonephros and connects secondarily with both. Torrey(2), in analyzing rete development in the albino rat, contended that the primordial rete differentiates from the gonadal blastema coincidentally with the sex cords, and only later becomes associated with the mesonephros. His position is thus somewhat unlike all the above, but is most closely allied with the second.

The present study on the chick embryo follows lines similar to those outlined by Torrey and is designed to resolve the conflicting opinions of this segment of avian development. It includes both a redescription of normal ontogeny of the rete and an experimental analysis.

For descriptive study, Barred Rock embryos and chicks were collected from the 96th hr of incubation to the 30th day after hatching. In the earlier stages, 4-9 days incubation, the embryos were collected at 8 hr intervals; from the 9th day of incubation to the 15th day after hatching, specimens were collected only at daily intervals; from the 15th-30th day after hatching, chicks were chosen at 2-3 day intervals. All of the material was fixed in Bouin's fluid, sectioned transversely at 10-15 μ, and stained either with Mallory's triple stain or Heidenhain's "Azan" modification of the Mallory method.

Normal ontogeny. The primordial, sexually undifferentiated rete first appears in embryos of 112 hr as strands of cells (Fig. 1) extending from the bases of the primary sex cords into the mesonephric stroma. From the beginning the rete is in structural continuity with and is interpreted as having a common origin with the primary sex cords. For the next 2 days there is little to distinguish males from females rete-wise, but in male embryos of 7 and 8 days the strands of rete testis will

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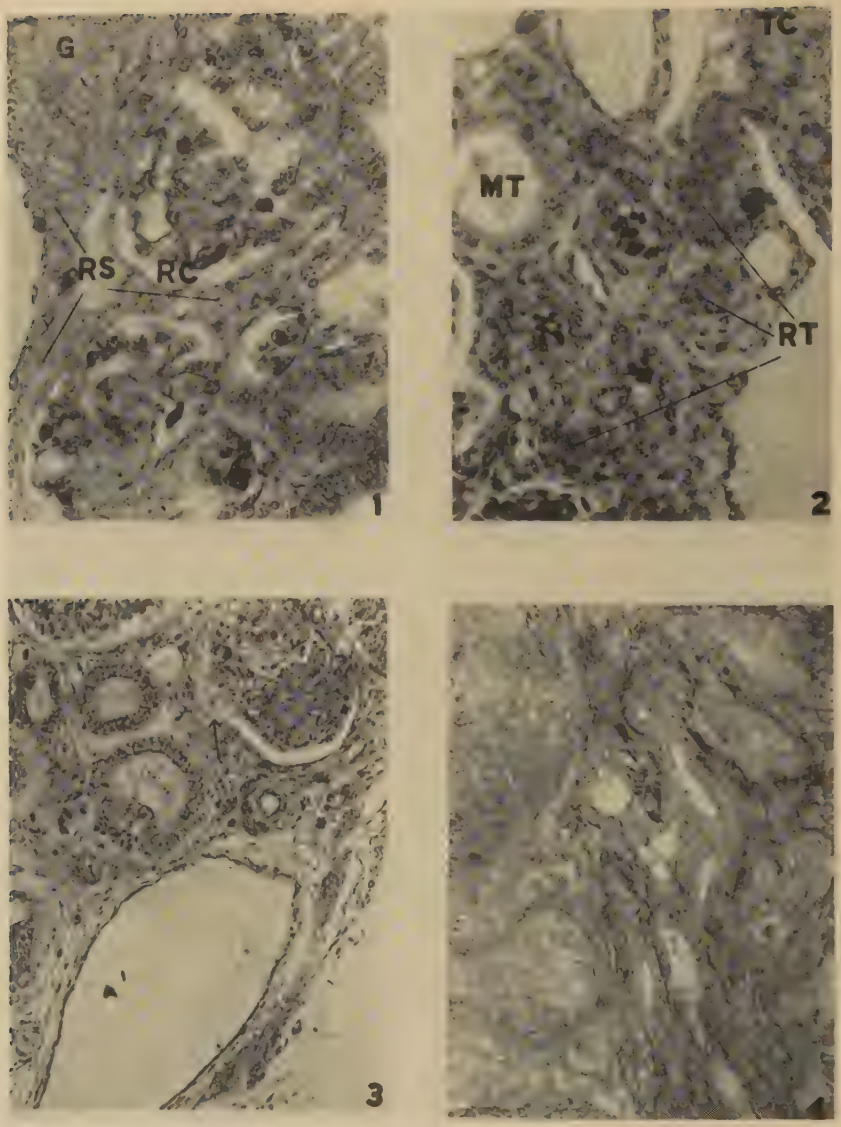

PLATE 1

FIG. 1. Strands of primordial rete (RS) projecting from gonad (G) and in contact with renal capsules (RC). 112-hr embryo. Mallory, × 200.

FIG. 2. Rete testis (RT) of 11-day embryo. Mesonephric tubule (MT); testis cord (TC). Note interruption of rete tissues by blood vessels. Mallory, × 200.

FIG. 3. Fusion of rete testis and walls of renal capsules. Arrow points to an area of fusion. 12-day embryo. Mallory, × 100.

FIG. 4. Initial cavitation of rete testis. 3-day chick. Mallory, × 200.

have become more bulky and by the 11th day subjected to considerable vascular invasion (Fig. 2). Fusion of rete testis and renal corpuscles is inaugurated by the 12th day and continues through hatching (Fig. 3). While morphological continuity from the potential tubuli recti of the testis through the rete to the potential epididymis is established by this fusion, at this time the rete and sex cords are solid. A few of the rete cords appear to blend with the nearby adrenal cortex rather than the renal corpuscles. Cavitation of the rete testis usually begins near the third day after hatching (Fig. 4), although sometimes

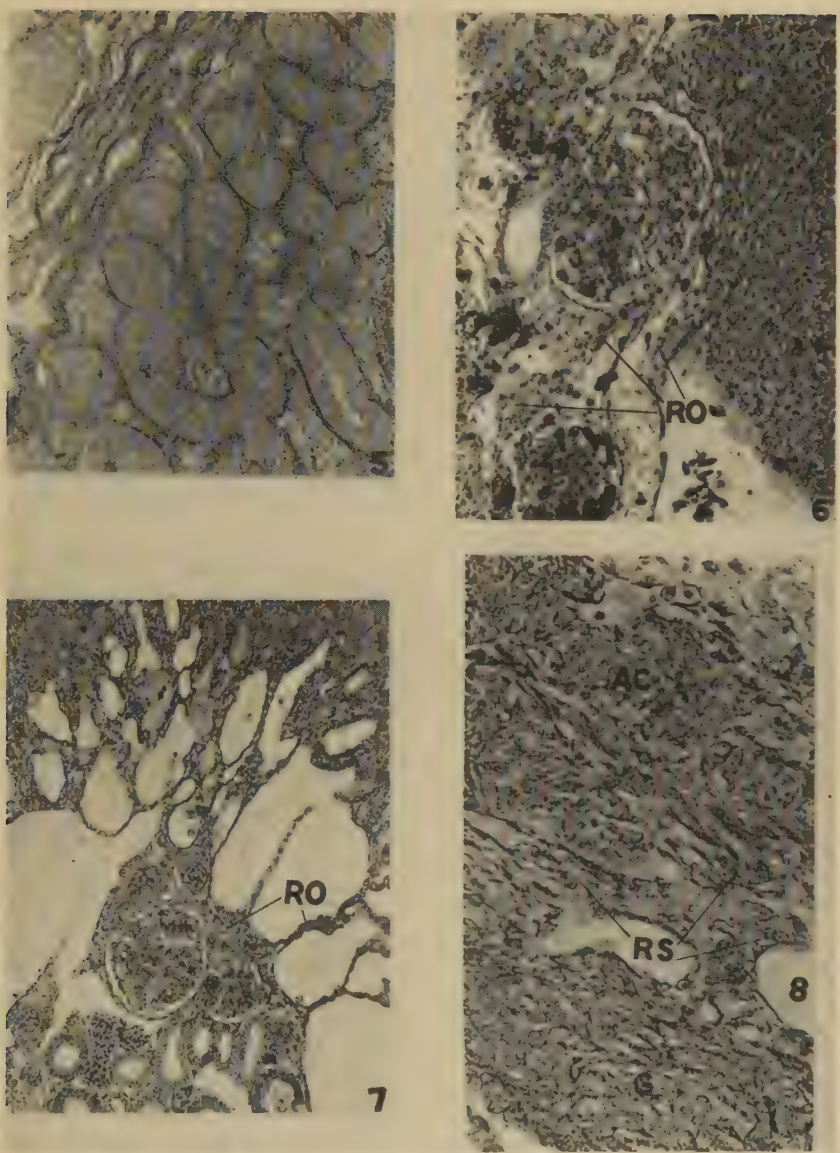

PLATE 2

FIG. 5. Rete testis of 3-week-old chick. Note continuity of rete tubule and sex cord. Mallory, × 100.
FIG. 6. Rete ovarii (RO) of a 7-day embryo. Mallory, × 100.
FIG. 7. " " " " " " " " × 100.
FIG. 8. Strands of primordial rete (RS) passing from gonad (G) towards adrenal cortex (AC). Mesonephros absent as result of block of wolffian duct. Mallory, × 100.

earlier; cavitation of the sex cords by the end of the third week after hatching. In the meantime the rete testis has become a system of broad, branching tubules with clean cavernous lumina (Fig. 5). With the establishment of the seminiferous tubules, the "open" system from the testis via the rete to the epididymis is complete. While the strands of cells constituting the rete ovarii are in 7-day embryos (Fig. 6) essentially like those of the rete testis, they are more tenuous and never become so bulky as in males. Shortly (176 hr), too, the ovary is characterized by degeneration and vacuolation among those medul-

lary cords which are adjacent to the mesonephros. This vacuolation of the medullary cords is accompanied by a further thinning and reticulation of the rete tissues. By the 9th day vacuolation of the deeper medullary cords has progressed so that at many points their walls are reduced to thin strings of cells. These in turn are continuous with the tenuous rete strands which pass into the mesonephros and fuse with the capsules of the renal corpuscles (Fig. 7). While the rete ovarii increases in bulk for the next few days and clearly blends with the renal corpuscles, it is never at any time so voluminous as in male embryos of corresponding age. Ultimately, too, the rete system will become virtually isolated in the mesovarium, for from the 12th day the connection of medullary and rete cords becomes more and more tenuous and within a day or two after hatching the junctions of rete and renal corpuscles have begun to break down as well. By the 15th day after hatching the vacuolated medullary cords will have disappeared almost entirely, and with their mesonephric connections now scanty, the rete cords, some showing incipient lumina since the 3rd day, are essentially isolated.

Experimental analysis. The experimental method used by Torrey(2) on the albino rat in which he cultivated separately the primordia of the gonad and of the mesonephros in the anterior chamber of the adult rat eye is not feasible for the chick embryo. In the chick, the association of the primordial gonad and mesonephros is too intimate to permit such a separation. However, several investigators have reported an operation in chicks which provides an excellent tool for this problem. Gruenwald(3) found, in agreement with Boyden(4) and substantiated by Waddington(5), that the differentiation of nephrogenic tissue into mesonephros took place only where that tissue was in contact with the wolffian duct. If a block or injury were made in such manner as to interrupt the backward growth of the wolffian duct, mesonephric tubules failed to appear, but, in the absence of a wolffian duct and mesonephros, the gonad developed normally. This is obviously highly significant, for if, in the absence of the mesonephros, it were found that the rete develops in association with the gonad, then the rete is shown to be gonadal in origin. On the other hand, if the rete does not appear, it may mean one of two things: either the rete is mesonephric in origin, or, is gonadal in origin but requires an inductive stimulus provided by the mesonephric tubules. Thirty-hour embryos of Barred Rock chickens were exposed via an opening sawed through the shell. An extremely fine glass needle was inserted to a depth of approximately one millimeter immediately caudal to the last distinctly formed somite on the left side of the embryo. When the needle was withdrawn, some yolk was allowed to extrude. This plug of yolk served as the block for the excretory duct. When the operation was completed, the opening was closed by an egg-shell cap. Because of a high mortality rate, experimentals were not recovered beyond the 6th day of incubation. This appeared to reflect a critical period of embryonic physiology at about the 96th hr of incubation, wherein any interference with the excretory system is especially deleterious. Recovered embryos were prepared for microscopical study in conventional fashion.

The experimental results fall into 2 groups: one set of 12 embryos which show a complete absence of one mesonephros, and a second set of 22 embryos which show a variably incomplete block of one mesonephros. Although the latter group does not provide a critical test for the problem at hand, it does give results worth discussion. Only typically illustrative cases will be described and analyzed.

A. *Complete block of mesonephros.* The first embryo selected for illustration was sacrificed after 112 hr of incubation, at which stage the rete normally appears. A well developed mesonephros with a normally developing gonad occurs on the right side of the embryo. By contrast, there is a large empty space on the left side. The left mesonephric body is entirely lacking; in fact, even the potential mesonephros, *i.e.*, nephrogenic tissue, is very much reduced. In spite of this lack of nephrogenic material, a well developed and normal gonad appears on the operated side. This gonad has an extensive and nor-

mally developed germinal epithelium sharply delimited by a basement membrane. Centrally small, cordlike masses of cells, the primary sex cords, appear, and from the bases of the latter, several distinct strands of cells (the incipient rete strands) pass out between the intervening blood vessels toward the developing adrenal cortex (Fig. 8).

Similar conditions are found in a slightly older embryo (120 hr). The left gonad projects as an ovoid mass and is clearly much larger than at 112 hr. The greater bulk of this gonad has resulted from proliferation of the primary sex cords in its central portion. From the bases of these cords and clearly continuous with them, rete strands flare outward to meet and blend with the cells and cordlike processes of the adrenal.

In a 6-day experimental embryo, the gonad is still larger and definitely spherical in shape. It has a well defined germinal epithelium which contains a number of germ cells. In the deeper portions of the gonad there are numerous, spherical and elongated primary sex cords, with an occasional germ cell present therein. Rete strands are present and project from the sex gland. They unite as before with the tissues of the adrenal bodies.

It is clear, then, that even in the absence of the mesonephros a normal gonad makes its appearance at the usual time and in the usual place, and that from the medullary cords of these "isolated" gonads rete strands project toward the adrenal cortex. It will be recalled that in the normal ontogeny of the rete a similar association between some of the rete cords and the adrenal cortex prevails. But whereas the bulk of the rete normally joins the mesonephric capsules, in these cases experimentally deprived of their mesonephroi all the rete strands run to the adrenal. The significance of this is difficult to assess, although one might speculate that the rete cords have a predilection for attachment to other bodies and in the absence of their normal attachment site, *i.e.*, the mesonephric capsules, seek out the nearby adrenal cortex instead. In this connection it is interesting that adrenocortical material has been described in association with the rete and epoophoron in the ovaries of such mammals as moles and squirrels(6) and pikas(7).

B. *Incomplete block of mesonephros.* In this series, the mechanical block of the wolffian duct was successful only in varying degrees; thus, all of these embryos show some traces of mesonephric differentiation. Since the amount and the particular location of the mesonephros varies greatly, separate descriptions of the differing situations are necessary before a general evaluation of the results can be made.

1. Mesonephric differentiation anterior to the gonad: In 4 embryos (with incubation ages from 120 to 144 hours) only 3-5 mesonephric tubules differentiated; 3 others (incubated 120-136 hours) have 6-10 tubules present. No renal corpuscles appear on the operated side in any of these embryos. Most importantly, the mesonephric differentiation which occurs is so far anterior as to leave the gonads isolated. In these "isolated" gonads, rete strands flare out from the ends of the primary sex cords and join the adrenal bodies.

2. Mesonephric differentiation posterior to the gonad: Three embryos (120 hours) have 3-6 tubules and one (136 hours) has 6-10 tubules which develop posterior to the gonads leaving them isolated as in the previous instance. One embryo shows a few renal corpuscles on the operated side; the others have none. In all of these "isolated" gonads, rete strands flare out from the ends of the primary sex cords and join the adrenal bodies.

3. Mesonephric differentiation anterior to and flanking the gonad: Seven embryos (aged 120-144 hours) have 3-10 mesonephric tubules on the operated side; 3 of them also have 2-4 renal corpuscles present. In these cases, the mesonephric differentiation flanks the gonad to some extent. In spite of this, rete strands are to be noted projecting from the gonad toward the neighboring adrenal tissue.

4. Mesonephric differentiation alongside the gonad: Two embryos (126 hours) have 2-5 mesonephric tubules and one embryo (136 hours) has 6-10 mesonephric tubules which develop somewhat laterally to the gonad. There is no trace of renal corpuscles in any of these cases. Rete strands are clearly discernible in the gonads of these embryos; they pass

out from the gonad toward the neighboring adrenal tissue.

5. Mesonephric differentiation both anterior and posterior to the gonad: In one embryo (120 hours) 12-15 mesonephric tubules appear together with 2 renal corpuscles. As all of the mesonephric differentiation occurs anterior and posterior to the gonad, the sex gland is again "isolated". Rete strands are clearly present and project toward the adrenal cortex.

In assessing these 22 cases showing mesonephric differentiation on the operated side, it should be noted that in all, a normal gonad with a full complement of rate strands developed in conjunction with an extremely small number of mesonephric tubules. The number of mesonephric tubules varied from 1-15. Now, if the mesonephric tubules or the renal corpuscles are the material source of the rete, then quantitatively there should be a corresponding deficiency of the rete. On the contrary, the rete on the operated side is always comparable to its counterpart on the unoperated side. Further, only 6 of the 22 cases have any renal corpuscles whatsoever on the operated side, yet rete strands are seen to develop and differentiate at a normal rate in all. These results, therefore, militate against a tubular or capsular origin for the rete.

Summary. The primordial rete of the chick embryo is a product of the gonadal blastema. It differentiates coincidentally with the primary sex cords with which it is continuous from the beginning. The rete testis is elaborated into a well vascularized network which invades the anterior portion of the mesonephros. There the rete tissues invest and ultimately fuse with the capsules of renal corpuscles. Cavitation of the rete testis begins within the first 3 days after hatching and is well advanced by the end of the 3rd week, when lumina appear in the sex cords. The rete ovarii in general parallels the development of the rete testis. However, its bulk is always less and shows only incipient cavitation. As a result of breakdown of the medullary cords accompanied by a disintegration of the connection between rete and renal capsules, the rete ovarii ends up as a rudiment isolated in the mesovarium. In embryos where a complete block of one mesonephros is effected experimentally, rete cords make their appearance associated and continuous with the sex cords of a normally differentiating gonad. In these cases, rete differentiation is independent of mesonephric differentiation. Embryos with the mesonephros only partially blocked on one side give evidence less critical but favorable to the view of gonadal origin of the rete.

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A Comparison of Fungistatic Properties of Three Aromatic Diamidines. (20547)

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The introduction of the aromatic diamidines as hypoglycemia-producing agents and the demonstration of their chemotherapeutic activity in protozoal diseases has led to experimental studies on their activity in the fields of bacterial, mycological, and neoplastic diseases. Favorable clinical results in patients suffering from blastomycosis(1,2), actinomycosis(3), and nocardiosis(2) have been reported following treatment with stilbamidine.

Observations following treatment with the aromatic diamidines in histoplasmosis(4-7), torulosis(8), and coccidiomycosis(9) have been variable. Studies on the *in vitro* effectiveness of the aromatic diamidines have been limited(1,10-12). Further studies of the specificities of the diamidines against certain pathogenic fungi might help to explain some of the discrepancies in the clinical observations.

Methods. Sabouraud's glucose agar was prepared with distilled water and autoclaved for 15 minutes at 15 lb pressure. The agar was cooled to 45°C and appropriate amounts of Seitz filtered stilbamidine (4,4′ stilbenedicarboxamidine), propamidine (p,p′-(trimethylenedioxy) (dibenzamidine) and pentamidine (p,p′-(pentamethylenedioxy) dibenzamidine)* were added. The concentrated aqueous solutions of the 3 drugs were Seitz filtered to sterilize, in view of the fact that in solution stilbamidine can be heated to 70-80°C for only a short time without decomposition(13) and it was felt that the chemical structures of the other 2 drugs were also prone to ready decomposition. The effect of amounts of 100 μg, 10 μg, and 1 μg of drug per ml of agar on the various fungi were tested. To obviate the effect of pH as noted by Elson(12), the pHs of different lots of the agar were adjusted to 7.4 following the addition of the diamidines. The fungi to be tested were initially grown on Sabouraud's glucose agar pH 7.5 for 28 days. The mycelial mass was removed aseptically with as little of the agar as possible and placed in a tube containing glass beads and 10 ml of sterile saline. This mixture of fungi and saline was shaken for 20 minutes in a Kahn shaker and 0.1 ml amounts of the mycelial-spore mixture were spread evenly over the surface of the agar slant containing the drug. The tubes were incubated in the dark for 30 days at room temperature and readings for inhibition were made at 4-day intervals. All of the *in vitro* tests were performed in triplicate and the presence of growth was determined by the use of the disecting microscope and cotton blue mounts. *Candida albicans, Cryptococcus neoformans, Histoplasma capsulatum, Blastomyces dermatitidis, Microsporum audouini, Microsporum canis, Tricophyton rubrum, Tricophyton tonsurans* (crateriforme) *Sporotrichum schenkii, Coccidioides immitis,* and a Madurella species were employed as test organisms.

The results of the *in vitro* tests are shown in Table I.

Our results with stilbamidine and *H. capsulatum* closely paralleled the observations of Seaburg(10) but *in vitro* the 0.01 mg/ml concentration of propamidine had some inhibitory effect suggesting it may be the drug of choice. In the case of *C. neoformans* and propamidine we found a close correlation with the value as determined by Fisher(4) for inhibition but pentamidine at the 0.1 mg/ml level displayed complete inhibition. Although our figures for the fungistatic properties of propamidine against *M. audouini, M. canis, T. rubrum, B. dermatitidis, C. albicans,* and *S. schenkii* do not correspond exactly with those of Elson (12), it was felt that the minor variations could be explained on the basis of pH and strain differences. It appears from Table I that the drugs differ in their capacity to inhibit certain fungi *in vitro.*

In view of our findings and the previously reported observations, it was decided to determine the effectiveness of the 3 diamidines against *Sporotrichum schenkii* infection in mice. This organism was chosen because of a slow progression of the disease with the production of grossly recognizable nodules. Twenty-one day-old Carworth white mice weighing between 22 and 25 g were inoculated intraperitoneally with 0.2 ml of a gastric mucin(13) suspension of *Sporotrichum schenkii* (BJC '52) grown at room temperature on Sabouraud's glucose agar. The mice were maintained on normal ration until the 26th day post infection at which time all mice showing gross subcutaneous nodules were isolated and separated into 4 groups. Groups 1 through 4 received stilbamidine, propamidine, pentamidine, and no therapy respectively. Three small groups of normal uninoculated mice served as controls on the effect of the diamidines alone. The drugs were in-

* We are indebted to Dr. Stephen Fromer, Merck & Co., and to Dr. R. C. Pogge, Wm. S. Merrell Co. for supplying the drugs used in this investigation.

TABLE I. A Comparison of the *In Vitro* Fungistatic Activity of 3 Aromatic Diamidines against Pathogenic Fungi.*

	Stilbamidine, mg/ml			Propamidine, mg/ml			Pentamidine, mg/ml			
	.1	.01	.001	.1	.01	.001	.1	.01	.001	Control
C. albicans	1+	2+	4+	2+	3+	4+	1+	3+	4+	4+
C. neoformans	4+	3+	4+	1+	4+	4+	0	4+	4+	"
H. capsulatum	0	4+	4+	0	2+	4+	4+	4+	4+	"
B. dermatitidis	0	0	2+	0	1+	3+	0	2+	4+	"
S. schenkii (BJC52)	0	1+	4+	0	0	2+	0	0	2+	"
M. audouini	4+	4+	4+	0	4+	4+	0	0	4+	"
M. canis	4+	4+	4+	4+	4+	4+	4+	4+	4+	"
T. rubrum	1+	3+	4+	0	0	4+	1+	2+	3+	"
T. crateriforme	3+	4+	4+	1+	2+	4+	2+	3+	4+	"
C. immitis	0	2+	4+	0	4+	4+	0	2+	3+	"
Madurella sp.	0	4+	4+	0	2+	4+	0	2+	4+	"

* 0 = complete inhibition; 4+ growth comparable to that of the controls.

corporated into the drinking water of the mice and were made up fresh daily in distilled water to a final concentration of 0.2 mg/ml. Observations indicated that groups of 4 mice ingest between 22-23 ml of water/day, thus it was assumed that each mouse would average 1.0-1.6 mg of drug intake in a 24-hour period. The mice were maintained on the drugs for a 20-day period and then observed for an additional 30 days. It was presumed that any evidence of therapeutic success would be accompanied by a remission of the subcutaneous abscesses, thus mice surviving to the end of the 76th day with no remission of the nodules were considered to be still actively infected. Mice dying on or before the 76th day were autopsied and cultures obtained from one or more of the abscesses. The results from a group of 175 mice failed to show any clear statistical evidence of therapeutic improvement. Mortality in the control groups was 13.8% and in the 3 groups on diamidine therapy ranged from 10.0 to 15.8%. Histological sections on the liver and kidneys of random selected surviving mice showed only slight degenerative changes† attributed to the increased toxicity of the diamidines which were exposed to light for 12-16 hours in the water bottles.

Discussion. The diamidine spectrum of 7 of the fungi causing systemic mycosis and 4 causing dermatomycosis have been examined. In general these findings have indicated some correlation with values obtained by other investigators. These figures indicate quantitative differences in the degree of inhibition of the 3 aromatic diamidines *in vitro*. Thus, the failure of certain of the diamidines to influence the clinical course of torulosis may be due in part to the relative ineffectiveness of the specific drug *in vitro* at levels that can be achieved in the blood stream. If these observations can be substantiated by other workers using different strains of the same fungi, it may become necessary to determine the drug sensitivities *in vitro* prior to instituting treatment.

The failure of the 3 aromatic diamidines to influence the course of *Sporotrichum schenkii* infection in mice may be due to an inability to achieve a sufficiently high level of the drug in the blood stream. This preliminary experiment was an effort to determine the effect of the drugs on sporotrichosis in mice, the possibility of an easier route of administration and the effect of maintaining a constant level of drug in the blood stream.

Summary. 1. The *in vitro* sensitivity of 11 fungi have been found to differ appreciably in the presence of stilbamidine, propamidine, and pentamidine. 2. The 3 diamidines appeared to have no therapeutic effect upon sporotrichosis in mice under the conditions of the experiment.

† The author is indebted to Dr. Melvin Haley, Dept. of Pathology, Baylor, for examination of the histological sections of the experimental mice.

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Failure of Choline Therapy to Alter Serum Lipids in Patients with Coronary Artery Disease.* (20548)

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The influence of lipotropic agents upon serum lipids has been studied extensively(1). Chemical studies in patients treated with these drugs over a prolonged period of time have been relatively few; in many of these investigations, the number of determinations have not always been sufficient to minimize the possible error due to spontaneous fluctuations of the serum lipids. For this reason, a long term study was undertaken.

Method. The present observations were made on 11 ambulatory patients, 10 males and one female, ranging in age from 28 to 57 years. Ten subjects had definite arteriosclerosis as evidenced by coronary insufficiency, or previous myocardial infarction, or both; the remaining patient was a member of a family with a striking family history of arteriosclerosis and was suspected of having coronary artery disease. The patients were studied for periods ranging from 6.5 to 15.5 months. Each consumed a self-selected diet. Before starting therapy, at least 3 chemical analyses were done in each case during a control period which varied from one to 5 months. Choline, which is considered the most effective lipotropic agent(2), was given orally in divided doses, as choline dihydrogen citrate (equivalent to 4.5 g of choline base daily), for periods ranging from one to 4 months. These were alternated with control periods ranging from one to 6 months. The serum cholesterol and serum lipid phosphorus were determined at the end of each choline period, at the end of each control period and also at intervals during these periods. The phospholipid:total cholesterol ratio (P/TC) was calculated according to the formula: Lipid P (mg %) x 25/Total Cholesterol (mg %). All determinations were done in duplicate; phospholipids were determined by a modified Whitehorn method(3) and cholesterol by the method of Schoenheimer-Sperry(4). Weight of the patients was recorded at intervals during the study.

Results. Table I summarizes the results obtained. It will be observed that choline therapy did not influence the levels of the total cholesterol and lipid phosphorus when compared to the control values. It is interesting to note that the mean during the control studies for lipid phosphorus was 11.1 mg %, total cholesterol 253 mg % and P/TC 1.10. Under choline therapy, the means for

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TABLE I. Average Levels of Serum Lipid Phosphorus (LP), Total Cholesterol (TC) and Phospholipid/Total cholesterol ratio (P/TC) in patients with coronary artery disease during control periods and while on choline therapy.

		Control periods					Choline therapy				
Sex and age	Diagnosis	Duration of study, mo	No. of assays	Avg LP, mg %	Avg chol., mg %	Avg P/TC	Duration of study, mo	No. of assays	Avg LP, mg %	Avg chol., mg %	Avg P/TC
♂, 53	Coronary insufficiency	7.5	7	13.4	372	.90	8	6	14.2	392	.90
♂, 48	Myocardial infarction	9	8	11.4	287	1.00	5.5	3	10.1	268	.94
♀, 40	"	8	9	10.7	246	1.08	7.5	5	10.3	241	1.07
♂, 28	Coronary insufficiency	8	6	9.2	217	1.06	2	1	8.4	218	.96
♂, 48	Myocardial infarction	7	7	9.5	211	1.12	7	5	9.1	219	1.04
♂, 40	Coronary insufficiency	6	6	11.6	236	1.23	3.5	2	11.2	246	1.13
♂, 43	Myocardial infarction	10	6	13.4	256	1.31	4	2	15.5	305	1.27
♂, 53	Coronary insufficiency	5	4	10.3	235	1.09	1.5	1	8.2	220	.93
♂, 46	"	5	4	11.7	264	1.11	1.5	1	13.6	270	1.26
♂, 57	"	5	4	12.2	261	1.17	1.5	1	13.9	255	1.36
♂, 52	No known heart disease	5	4	9.0	194	1.16	1.5	1	11.3	212	1.33
Mean				11.1	253	1.10			11.4	259	1.10

these lipid values were strikingly similar; the lipid P was 11.4 mg %, the total cholesterol 259 mg %, and the P/TC 1.10. During the entire period of observation, there were no significant alterations in body weight. It may be of interest to record that case No. 1, a male aged 50, with a severe anginal syndrome, developed an acute myocardial infarction while on choline therapy.

Conclusion. The oral administration of 4.5 g of choline base daily for 1.5 to 8.0 months to 11 patients with coronary insufficiency or myocardial infarction or both, failed to alter the total serum cholesterol, the serum lipid phosphorus and the phospholipid:total cholesterol ratio.

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Steroids in Adrenal Venous Blood of the Dog.*† (20549)

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Adrenal venous blood of the dog has been quantitatively analyzed for steroid components. Fourteen fractions have been isolated, 10 of which appear to be steroids. Three have been identified as 17-hydroxycorticosterone, corticosterone, and 11-desoxy-17-hydroxycorticosterone. Certain chemical characteristics of the remaining fractions have been determined.

Methods. Male dogs were anesthetized with sodium pentobarbital. Adrenal venous blood was collected from a cannula placed in the left lumboadrenal vein. All collateral branches of the lumboadrenal vein were ligated and the adrenal vein ligated between the gland and the inferior vena cava. Thirty to 50 mg heparin were injected in divided doses to prevent clotting. Blood was collected in lots of approximately 250 ml. Immediately after collection, each lot was diluted 1:1 with water and extracted twice with a volume of chloroform equal to that of blood plus water. Centrifugation was necessary to break the emulsion formed during extraction. The pooled chloroform extracts from blood of a single dog were evaporated to dryness immediately or kept overnight at —10°C and evaporated the next day. A total of 3500 ml of adrenal venous blood was obtained from 4 dogs; the dried extracts were pooled and partitioned between hexane and 70% ethanol. The 70% ethanol fraction was chromatographed on paper by the method of Burton *et al.*(1) using the solvent system propylene glycol-toluene. An outline of the design of chromatography is presented in Table I. The developed chromatograms were examined for steroids as follows: 1) the entire strip was divided at right angles to its length into a series of ½- to 1-inch cuts which were eluted in methanol in individual test tubes and subsequently analyzed, or 2) color tests (reaction with ammoniacal $AgNO_3$, triphenyltetrazolium, or concentrated sulfuric acid streaked on a glass plate) were applied to narrow strips cut the full length of the chromatogram and those portions of the chromatogram corresponding to the indicated locations of steroids were cut out and eluted in methanol for further analysis. The first method was used in screening preliminary chromatograms; the second, in isolating individual fractions. The eluted substances were quantitatively analyzed by 4 methods: 1) ultraviolet absorption spectrum, 2) reaction with phenylhydrazine, 3) reaction with concentrated sulfuric acid, and 4) reaction with m-dinitrobenzene.

Results. Fourteen fractions were isolated. (Tables I and II). *Fraction 6* chromatographed in the same area as 17-hydroxycorticosterone. Ultraviolet absorption, 240 mμ; reaction with sulfuric acid, orange color with strong fluorescence; absorption in phenylhydrazine reagent to give the 400 mμ peak characteristic of 17,21-dihydroxy-20-ketosteroids; absorption maxima at 240, 280, 390, and 470 mμ in concentrated sulfuric acid. On the basis of these findings, Fraction 6 was identified as 17-hydroxycorticosterone. Its concentrations, 147 μg/100 ml adrenal venous blood, was the highest of the various fractions isolated.

Fractions 1 through 5 were more polar than 17-hydroxycorticosterone. A narrow strip cut from Chromatogram 3 and placed on a glass plate streaked with concentrated sulfuric acid exhibited the following bands of color starting at the origin and progressing toward the front: 1) green fluorescence, 2) pink, 3)

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TABLE I. Paper Chromatography of Extract of Adrenal Venous Blood (Propylene Glycol Stationary Phase, Toluene Mobile Phase).

3500 ml adrenal venous blood

↓

Combined $CHCl_3$ extracts partitioned, 70% ethanol : hexane

↓

Chromatogram 1—72 hr, HSPG*: toluene, 70% ethanol fraction. The first inch, heavily pigmented, was discarded; the remainder was eluted and rechromatographed.

↓

Chromatogram 2—72 hr, HSPG : toluene, eluate of Chromatogram 1. Components incompletely resolved. Chromatogram divided.

↓

Chromatogram 3—240 hr, HSPG : toluene, first 2 inches of Chromatogram 2. Fractions 1 through 6 resolved.

↓

Chromatogram 6—15 hr, HSPG : toluene, overflow of Chromatogram 1. Components incompletely resolved.

↓

Chromatogram 7—22½ hr, HSPG : toluene, eluate of Chromatogram 6. Incomplete resolution.

↓

Chromatogram 8—23½ hr, FSPG*: toluene, eluate of Chromatogram 7. Fractions 9 and 10 incompletely resolved. Fractions 11 and 12 resolved.

↓

Chromatogram 9—48 hr, HSPG : toluene, fractions 9 and 10 from Chromatogram 8. Fractions 9 and 10 resolved.

Chromatogram 4—144 hr, HSPG : toluene, remainder of Chromatogram 2. Fractions 6 and 7 resolved.

↓

Chromatogram 5—96 hr, HSPG : toluene, overflow of Chromatogram 4. Fraction 8 isolated.

↓

Chromatogram 10—5½ hr, FSPG : toluene, combined overflows from Chromatograms 6, 7 and 8. Fractions 12 and 13 incompletely resolved. Fraction 14 isolated.

↓

Chromatogram 11—24 hr, FSPG : toluene, fractions 12 and 13 of Chromatogram 10. Fractions 12 and 13 resolved.

* HSPG = half-strength propylene glycol. Propylene glycol is diluted 1:1 with methanol before application to the paper and the methanol allowed to evaporate.
FSPG = full-strength propylene glycol.

green fluorescence, 4) green fluorescence and 5) pinkish brown. Immediately below Fraction 5 was the brilliant green fluorescence of Fraction 6 (17-hydroxycorticosterone). Fractions 1, 3, and 4 reduced ammoniacal $AgNO_3$ and reacted with phenylhydrazine; they exhibited no definite maxima at 240 mμ. Fractions 2 and 5 absorbed in the ultraviolet but did not react with phenylhydrazine. Fraction 2 absorbed maximally at 255 mμ, and 5, at 285 mμ. Concentrations were estimated on the basis of the phenylhydrazine reaction: Fraction 1, 1.25; Fraction 3, 3.8; Fraction 4, 1.0 μg/100 ml. Fractions 2 and 5 could not be quantitated.

Fraction 7. About 5 μg/100 ml of this unidentified steroid were present. It reduced ammoniacal $AgNO_3$, absorbed at 230 mμ and reacted with phenylhydrazine reagent to give the 340 mμ maximum characteristic of Δ^4-3-ketosteroids without the 17,21-dihydroxy-20-ketone grouping. It gave an orange solution without fluorescence which absorbed maximally at 240, 285 and 370 mμ in concentrated sulfuric acid. Ultraviolet absorption, phenylhydrazine reaction and $AgNO_3$ reduc-

TABLE II. Substances Found in Adrenal Venous Blood of the Dog.

Fractions in order of polarity	Ammoniacal $AgNO_3$ reduction	Ultraviolet absorption (max. mμ)	Absorption in phenylhydrazine reagent (max. mμ)	Concentration (μg/100 ml)	Identification
1	+	—	400	1.25	Not identified
2	—	255	0	†	" "
3	+	—	400	3.80	" "
4	+	—	400	1.0	" "
5	—	285	0	†	" "
6	+	240	400	147.0	17-hydroxycorticosterone
7	+	230	340	5.0	Not identified
8	+	230–240*	400	3.0	" "
9	—	0	0	†	" "
10	—	215, 265	0	†	" "
11	+	240	400	15.0	11-desoxy-17-hydroxycorticosterone
12	+	240	340	47.0	Corticosterone
13	+	235	340	16.0	Not identified
14	?	230–235	330–340*	18.0	" "

* Inflection but no definite maximum.
† Quantitation not possible.

tion suggest the presence of a $\triangle^4$-3-ketone configuration and a α-ketol side chain. However, the presence of 2 more atoms of oxygen on the steroid nucleus is suggested by the position of this steroid on the chromatogram.

Fraction 8 was present in a concentration of about 3 μg/100 ml and chromatographed in the same region as cortisone. It reduced $AgNO_3$ and exhibited a maximum at 400 mμ with phenylhydrazine. The ultraviolet absorption curve was suggestive of absorption in the region 230 to 240 mμ. In concentrated sulfuric acid, the substance exhibited maxima at 285 and 390 mμ (cortisone exhibits maxima at 280, 340 and 400 mμ). The steroid did not react with iodine reagent (cortisone gives a brilliant blue color when treated with this reagent).

Fractions 9 and 10. Two unidentified substances were found whose polarity was slightly less than that of Fraction 8. The more polar of these, Fraction 9, became distinctly pink when treated with sulfuric acid, and exhibited maxima at 270, 410 and 485 mμ with this reagent; it did not absorb in the ultraviolet or react with phenylhydrazine. $AgNO_3$ reduction was questionable. Fraction 9 is probably not a steroid. Fraction 10 was slightly less polar than Fraction 9. It was seen as an absorbing band when the chromatogram was viewed under ultraviolet light. The material did not reduce $AgNO_3$, but rather it retarded the usual reduction which occurs when the paper strip, freshly dipped in ammoniacal $AgNO_3$, is exposed to light. It absorbed at 215 and 265 mμ and exhibited maxima at 210, 270 and 550 mμ with sulfuric acid. It did not react with the phenylhydrazine reagent. Fraction 10 is probably not a steroid.

Fraction 11, present in a concentration of about 15 μg/100 ml, was tentatively identified as 11-desoxy-17-hydroxycorticosterone. It reduced $AgNO_3$, absorbed at 240 mμ and gave a 400 mμ peak with phenylhydrazine. It became pink when treated with sulfuric acid, and the sulfuric acid chromogen was identical with that of 11-desoxy-17-hydroxycorticosterone. A mixture of 11-desoxy-17-hydroxycorticosterone and Fraction 11 chromatographed as a single spot.

Fraction 12 found on chromatograms 8, 10 and 11 was identified as corticosterone. It reduced $AgNO_3$, absorbed at 240 mμ, gave a brilliant green fluorescence with sulfuric acid and reacted with phenylhydrazine to give a maximum at 340 mμ. The sulfuric acid chromogen was identical with that of corticosterone. It was present in a quantity of 47 μg/100 ml, the second highest concentration of the fractions isolated.

Fraction 13 has not been identified. It re-

duced $AgNO_3$, absorbed at 235 mμ, gave a 340 mμ maximum with phenylhydrazine reagent and exhibited maxima at 280 and 370 with sulfuric acid. It was less polar than corticosterone, but considerably more polar than desoxycorticosterone. These data suggest a C_{21} O_4 compound with Δ^4-3-ketone and α-ketol groupings. The sulfuric acid chromogen was not the same as that of 11-dehydrocorticosterone (maxima at 280, 350 and 410 mμ). On the basis of the phenylhydrazine reaction, 16.0 μg/100 ml were estimated to be present.

Fraction 14 chromatographed close to the solvent front. The high mobility of this material in the solvent system precluded adequate resolution. It had a weak reducing effect on ammoniacal $AgNO_3$, absorbed strongly in the ultraviolet at 230 to 235 mμ and exhibited a maximum at 290 mμ in sulfuric acid. The absorption curve in the phenylhydrazine reagent exhibited an inflection but not a definite maximum in the region 330 to 340 mμ. Fraction 14 did not give a reaction with m-dinitrobenzene characteristic of 17-ketosteroids. If the ultraviolet extinction coefficient is of the same order of magnitude as that of the cortical steroids, Fraction 14 is present in a concentration of 18 μg/100 ml.

An eluate of that portion of Chromatogram 10 which would have contained substances less polar than Fraction 14 and the overflow of Chromatogram 10 were analyzed in the ultraviolet region of the spectrum and tested with m-dinitrobenzene. There was no evidence for the presence of Δ^4-3-ketones or 17-ketosteroids.

Discussion. Reich *et al.*(2) demonstrated the presence of 17-hydroxycorticosterone and corticosterone in dog adrenal venous blood. Bush(3) found 17-hydroxycorticosterone, corticosterone and 2 substances more polar than 17-hydroxycorticosterone. He depended largely upon position on the paper chromatogram for identification. Zaffaroni and Burton(4) reported the presence of 9 $AgNO_3$-reducing substances in dog adrenal venous blood. Two of these were identified as 17-hydroxycorticosterone and corticosterone; the other 7 were characteristized as α-ketols. The results presented in this paper confirm that 17-hydroxycorticosterone and corticosterone are the 2 major components present in dog adrenal venous blood. 11-Desoxy-17-hydroxycorticosterone has been identified as a component of adrenal venous blood for the first time. Eleven other fractions have been isolated and partially characterized and it appears that 7 of these are steroids. It is not certain that all these substances are produced by the adrenal. A definitive answer must await analyses of the concentrations of these substances in both arterial and venous blood of the adrenal.

The physiological significance of the complex pattern of steroids in adrenal venous blood cannot be evaluated at this stage of development of the problem. Each fraction must be assayed for biological activity regardless of its concentration as determined by chemical methods. Furthermore, the fractions must be assayed alone and in combination to test the possibility that the steroids in adrenal venous blood act synergistically or antagonistically. The biological activities of 17-hydroxycorticosterone, corticosterone and 11-desoxy-17-hydroxycorticosterone have been described. Fraction 8, present in relatively small concentration, has been demonstrated recently to exert a very potent sodium-retaining effect in adrenalectomized rats(5). Fraction 8 appears to be approximately 25 times as potent as DOCA and may be identical with the sodium-retaining factor recently described by Simpson *et al.*(6). This fraction is an example of a substance, present in very small amount in adrenal venous blood, which nevertheless exerts a very marked biological effect.

The adrenal cortex may exert a variety of metabolic effects by secreting a variety of patterns of adrenocorticosteroids. An aberration in electrolyte metabolism may be associated with the secretion of a pattern with a higher than normal concentration of those steroids whose predominant influence is on electrolyte metabolism. On the other hand, an aberration in carbohydrate metabolism may be associated with the secretion of a pattern with a higher than normal concentration of those steroids whose predominant in-

fluence is on carbohydrate metabolism. An approach to this problem is made possible by the development of technics for the quantitative analysis of steroids in adrenal venous blood.

Summary. Adrenal venous blood of the dog has been quantitatively analyzed by paper chromatography for steroid components. Fourteen fractions have been isolated; 3 have been identified as 17-hydroxycorticosterone, corticosterone and 11-desoxy-17-hydroxycorticosterone; 7 of the unidentified fractions exhibit chemical reactions characteristic of adrenocorticosteroids.

ADDENDUM. In a subsequent experiment conducted in collaboraton with Dr. Hans Hirschmann a sample containing Fractions 7 and 8 was acetylated and chromatographed on paper 90½ hours, employing the system propylene glycol-hexane described by Savard. Five distinct zones were found by examining the strip under ultraviolet light. These subfractions (numbered 1-5 in order of their position on the chromatogram, starting at the origin) were eluted with methanol and were examined spectroscopically in this solvent and after reaction with phenylhydrazine-sulfuric acid reagent. Subfractions 1, 2, and 3 absorbed maximally near 240 mμ; subfraction 4, near 235 mμ, while subfraction 5 showed only a shoulder in this region. With phenylhydrazine reagent subfractions 1 and 5 absorbed maximally at 390-400 mμ, whereas subfractions 2, 3, and 4 gave products which did not exhibit absorption peaks in this region. Each subfraction was treated with acetylcholinesterase in glycylglycine buffer at pH 7.5 for 4 hours at 30°C, and assayed for sodium-retaining activity by the method described in an earlier communication(5). All subfractions were inactive except subfraction 4 which possessed high sodium-retaining activity. No claim of homogeneity is made for any of these subfractions, but it would appear that the sodium-retaining activity and the chromogenicity in the phenylhydrazine reagent shown by our previously described Fraction 8 are not due to the same substance.

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Increased Excretion of Dehydroascorbic and Diketogulonic Acids in Urine of Rats after Standardized Temperature Shock. (20550)

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Increased urinary excretion of 2 oxidation products of ascorbic acid (AsA), namely dehydroascorbic acid (DHA) and diketogulonic acid (DKA) has been observed in rats during exposure to an environmental temperature of 0°C(1) and after exposure to high doses of X-ray(2), as compared to control excretions on comparable rats under normal conditions. A third type of stress, namely standardized temperature shock(3), has been tested and the results observed are reported here.

Experimental. The experimental animals were adult albino rats of both sexes, belonging to the same strain as those employed in the previous work(1,2), which were housed in individual metabolism cages as previously described. At the beginning of the control period, each animal was anesthetized with nembutal, and immersed up to the neck in a water-bath maintained at 37°C for 15 seconds.

TABLE I. Urinary Excretion of Ascorbic, Dehydroascorbic and Diketogulonic Acids by Rats before and after Severe Temperature Shock.

Fraction	No. of analyses: Control	No. of analyses: Temperature shocked	Avg 24 hr excretion, μg with S.D.: Control	Avg 24 hr excretion, μg with S.D.: Temperature shocked	"F"
AsA	99	87	441 ± 136	485 ± 178	3.7
DHA	99	87	2 ± 5	43 ± 39	112 *
DKA	99	87	0 ± 0	48 ± 41	79 *

* Probability of variance ratio, "F," less than .01.

The animals were then placed in their individual cages and the excretion of AsA, DHA and DKA was determined each 24-hour period for 7 to 10 days following the mock temperature shock by the method previously employed(4). When the control excretion for each animal had been established, the experimental period began. Each animal was anesthetized with nembutal, and immersed to the neck in a water-bath held at 60°C for 15 seconds; the animals were replaced in their cages and the daily excretion of AsA, DHA and DKA determined for 7 to 10 days following the temperature shock. There were no fatalities during this period.

Results. The results obtained are summarized in Table I. As with cold environment and X-ray irradiation, exposure to this degree of temperature shock caused a marked increase in urinary excretion of DHA and DKA, which is highly significant. There was a slight increase in AsA excretion, which did not attain the level of significance.

Discussion. It appears that this third type of stress, which differs from the 2 previously reported in physiologic mechanism, has the same effect of increasing excretion of oxidized ascorbic acid in the urine. The physiologic mechanism responsible for this remains unknown. Attempts to demonstrate the adrenal glands and the kidneys as the site of formation have been unsuccessful(5).

Summary. 1. Adult albino rats were subjected to temperature shock by immersion in water at 60°C for 15 seconds. 2. Urinary excretion of dehydroascorbic and diketogulonic acids increased greatly over control values established before temperature shock.

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Cortisone on Oxygen Consumption of Granulation Tissue from the Rabbit. (20551)

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It has been shown that cortisone and adrenocorticotrophin (ACTH) produce an inhibitory effect on growth of granulation tissue in rabbits(1) and around turpentine abscesses in rats(2); that ACTH causes a retardation of wound healing in humans as studied by serial biopsies(3). Histological studies of the latter reveal a lack of fibroblasts, capillaries and ground substance; a condition not unlike that found in scorbutic animals(3). This suggests an inhibition of anabolic processes which are of primary importance in tissue repair. The literature reveals no data exist relative to the action of cortisone, ACTH, or adrenocortical extract (ACE) on the metabolism of granulation tissue. This report in-

dicates studies directed toward this end.

Materials and methods. Fifteen young male white rabbits weighing between 2.5 and 3.5 kg were divided into 3 groups. They were kept under laboratory conditions (20-27°C) for at least one week prior to experimental use. They were fed Pioneer Rabbit Food and water *ad libitum.* In the first 2 groups (A and B) granulation tissue was produced by sterile removal of a 9 cm^2 flap of shaved dorsal skin to a depth of 0.035-0.050 inches by means of a Brown electric dermatome.* The absence of hair follicles was considered indicative of sufficient penetration into the dermis. After the operation the area was covered with sterile vaseline gauze and a plaster cast. Group C, uninjured untreated control animals were subjected to a sham operation which consisted of shaving, use of vaseline gauze and a plaster cast comparable in size and location to the other experimental groups. Groups A and C had no further treatment; Group B received 25 mg of a stabilized saline suspension of 11-dehydro-17-hydroxycorticosterone acetate (Cortone, Merck) per day starting 3 days prior to injury and ending the day before tissue respirometry. All experimental animals were sacrificed 6 days after, either the surgical or sham procedure, by the injection of 50 cc of air into the marginal ear vein. Uninjured untreated control animals (Group C) were operated on immediately following sacrifice in order to obtain "normal" dermis. The granulation tissue or dermis was carefully dissected from the hypodermis. Various areas of the tissue were sliced by means of a Warburg cutter, the slices varied in thickness from 0.4 to 0.9 mm with a mean of 0.65 mm. This procedure was carried out on a chilled glass stage (3-5°C). The oxygen consumption of pooled slices was determined by the Warburg technic; at least 6 manometers were run on a given sample. The manometers were oscillated through an amplitude of 4 cm at a rate of 110 cycles/minute. Each respiration flask contained 0.2 ml 10% oxygenated NaOH solution in the central well, 2.8 ml oxygenated Krebs-phosphate (pH 7.4) solution(4) plus 65-100 mg of tissue in the main chamber. The manometers were gassed with 100% O_2 for 10 minutes and equilibrated at 37.5° ± 0.01°C for 15 minutes. The elapsed time between sacrifice of the animal and equilibration of the manometers was *ca* one hour. Manometric readings were taken for 60 minutes at 15-minute intervals. Samples of pooled tissue from each of the 3 groups were taken for wet and dry (17-20 hours at 105-110°C) determinations. The mean percent value for water content of given tissue samples was used to standardize the oxygen consumption in terms of QO_2, *i.e.*, mm^3 O_2 consumed/hour/mg (dry) of tissue.

TABLE I. Summary of Data for Oxygen Consumption (QO_2) of Dermis, "Normal" and Cortisone-Treated Granulation Tissue.

Groups	Untreated injured (A)	Cortisone treated injured (B)	Untreated uninjured control (C)
No. of animals	6	5	4
No. of exp.	52	32	22
True mean	−1.34±.06	−.45±.03	−.27±.02
% diff. compared to control dermis (C)	+396%	+67%	
SD	.46	.19	.10
P-value	<.01	<.01	

True mean = arithmetic mean ± its stand. error; SD = stand. deviation of indicated mean; P-value = probability value. Statistical analysis based on Student's method, where t = difference between a given pair of means/estimated stand. error of this difference.

Results. The various data for the oxygen uptake of granulation tissue (Group A and B) and dermis (Group C) are summarized in Table I. The true mean QO_2 values for the 3 groups are: (A) —1.34 ± 0.06, (B) —0.45 ± 0.03, and (C) —0.27 ± 0.02. A comparison of the mean QO_2 values for Groups A and B with C (control) shows that "normal" granulation tissue (A) gives a 396% increase ($P < 0.01$) and cortisone-treated granulation tissue (B) a 67% increase ($P < 0.01$). Cortisone-treated granulation tissue (B) shows a 66% decrease ($P < 0.01$) when compared with non-treated granulation tissue.

Discussion. Dermal slices from normal

*We are indebted to Dr. E. H. Ellison of the Department of Surgery, Ohio State University Hospital, for use of the dermatome.

rabbits give a QO_2 of *ca* 0.3 under the experimental conditions prevailing. This may be compared with the only other known value of 0.9 for "strips of skin" of very young rats(5). The difference between these two values may be due to a species variation; however, "strips of skin" included epidermis which was absent in our study. This may suggest that a considerable difference exists between the oxygen consumption of epithelial and connective tissues. As expected there is an increase (+396%) in the endogenous metabolism of granulation tissue (Group A) compared with dermis (Group C) from the corresponding area of rabbits. The metabolism and water content of young "normal" cells are consistently higher than of older cells(6). However, the histological organization of granulation tissue and of dermis is not exactly comparable. The chief difference is not only smaller numbers of actively proliferating cells and capillaries, but also the almost complete absence of polymorphonuclear leucocytes in the latter. Therefore, it is suggested that the increase in oxygen consumption may be due not only to the presence of larger numbers of younger cells, but to different cell types found in granulation tissue as compared with dermis. A comparison of the oxygen uptake of cortisone-treated (Group B) with non-cortisone treated granulation tissue (Group A) shows a significant decrease (66%). This suggests that cortisone exerts an inhibitory action on the oxygen consumption of granulation tissues. Since this tissue is composed of various cell types, it further suggests that cortisone may inhibit the growth (anabolic) processes in one or more of the component cells, *e.g.*, fibroblasts, clasmatocytes or endothelial cells. It has been shown that whole adrenocortical extract (ACE) and corticosterone, when incubated with liver and brain slices from normal rats, inhibit oxygen consumption(7). Thus it appears that the cortisone fraction of ACE is probably not specific in its metabolic depressant action. The mechanism of this inhibitory action has not yet been elucidated. Further experiments are underway in this laboratory.

Summary. The oxygen consumption (QO_2) of pooled dermal and granulation tissue slices from 2.5-3.5 kg male white rabbits were studied by the Warburg technic. The rabbits were divided into 3 groups: (A) Those subjected to dermal injury. (B) Those subjected to dermal injury, but in addition receiving pre- and post-operative cortisone treatment, *i.e.*, 25 mg/day. (C) Those animals subjected to a sham operation. 1. "Normal" uninjured dermal slices give a mean endogenous QO_2 value of 0.27 $\pm$ 0.02 (Group C, Table I). 2. Slices from untreated granulation tissues (Group A) show an increase of 396% over their control (Group C). 3. Slices from cortisone-treated granulation tissue (Group B) give only a 67% increase over the control (C), or a 66% decrease when compared with untreated granulation tissue(A). 4. The implications of these results are discussed.

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Effect of Sodium Salicylate Treatment on Antibody Titers in Rats Infected with *Trypanosoma lewisi.* (20552)

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An increased parasitemia and a prolongation of the reproduction phase of *T. lewisi* in salicylate-treated rats has been described by Becker and Gallagher(1). The mode of action of salicylates, as it affects the immune response, has not been adequately explained. Two possible mechanisms of salicylate activity have been postulated, namely, an ACTH-like effect(2), or an interference with antigen-antibody reaction(3,4). This present investigation is an attempt to show that the circulating antibody concentration is lowered by salicylate treatment, lending support to the former viewpoint.

Materials and methods. Male Wistar rats 6 weeks old were infected intraperitoneally with the Becker strain of *T. lewisi* and divided into 2 groups: (A) untreated; (B) sodium salicylate in distilled water (90 mg/ml) administered daily from the day of infection via a stomach tube (45 mg/100 g body wt). Treatment was discontinued the day before sacrificing. The animals were sacrificed at various intervals (Table I) by exsanguination under anesthesia. Each antiserum was titrated for agglutinins using trypanosomes collected from untreated rats with an 8-day infection. The organisms were suspended in a phosphate buffered (pH 7.4) saline solution containing 0.002 M glucose and used at a concentration of 120 x 10^5 trypanosomes per ml in the titrations. All tubes were read at the end of a 2-hour incubation at room temperature. Antisera salicylate levels were determined by the method described by Keller(5).

Results. As may be seen in Table I demonstrable agglutinins appeared in untreated animals in most instances after the 8th day of infection. A parallel series of salicylate-treated animals did not develop agglutinins until the 30th day with the exception of a low titer in one animal at the 16th day. Many of the salicylate-treated animals died during the course of the experiment showing a high parasitemia at the time of death.

The concentrations of salicylate in the antisera were found to be low and unrelated to the agglutinin titers. Levels of salicylate ranged from 0.02 to 0.85 mg/ml in individual animals. To check the possibility of inhibition of the antigen-antibody reaction *in vitro* by these levels of salicylate, varying amounts of sodium salicylate were added to several antisera from untreated animals. No lowering of the agglutinin titers of these antisera was observed at concentrations of 0.3, 0.6, 1.2, 2.4, 4.8, and 9.6 mg/ml.

Discussion. The typical course of infection as described by Taliaferro(6) in untreated animals, and as altered by salicylate treatment(1) was observed in this study. Increased output of ACTH in animals receiving salicylate has been given as an explanation for the specific direct or indirect effect of the drug. Adrenal ascorbic acid depletion was not found in hypophysectomized animals receiving salicylate(2). The evidence that ACTH and cortisone treatment reduces antibody concentration seems to be adequate (7-9).

In this study a direct determination of the concentration of circulating antibody was carried out. Inhibition of antibody production or its increased catabolism, or both, could account for the lower agglutinin titers found in salicylate-treated animals. Lysenko has demonstrated a lowered total serum nitrogen and gamma-globulin in salicylate-treated infected animals(10). The progressive nature of the infection in treated animals may be the result of lowered antibody concentration.

Friend(11) and others have shown that salicylates inhibit antigen-antibody reactions *in vitro*. Friend obtained only 4% inhibition of an egg albumin-anti-egg albumin system using 80 mg of sodium salicylate per ml. This property of the drug was shown not to be operative in this investigation since salicylate

TABLE I. Agglutinin Titers of Untreated and Salicylate Treated Animals.

Day of infection	Neg.*	Serum dilution 1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	Avg mg sal./ml antiserum
					Untreated						
2	3										
4	4										
6	4										
8	2	1	1								
10		1	1	1							
12	1			3							
14	2		2								
16				1	1		1	1			
18		1				1	1		1		
20					1						
22			2	1		1					
26							1			1	
30							2	1		1	
35						2	1	1			
50						1					
60					1	2					
80				1							
90							1				
120								2			
170					1	1					
					Salicylate treated						
2	2										.83
4	4										.10
6	4										.09
8	4										.09
10	4										.27
12	5										.27
14	4										.23
16	3		1								.25
18	4										.41
20	2										.35
22	4										.28
26	2										.15
30	1		1					1			.43
35	3					1	1				.15
50					1	1	1				.23
60											
80					1	1					.12
90			1								.03

* No. of animals showing no agglutination.
Figures represent No. of individual rats tested.

levels in antisera were comparatively very low and the addition of salicylate to antisera from untreated animals showed no agglutination inhibition.

Summary. Daily treatment with sodium salicylate reduced the concentration of circulating antibody in rats infected with *T. lewisi*. Possible modes of action are discussed.

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Failure of Short-term Administration of Thyrotrophic Hormone to Produce Exophthalmos in Man.* (20553)

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It is widely held that thyrotrophic hormone (TSH) is the cause of human exophthalmos, but this has never been directly proven by experimental observation. This opinion is based upon the development of exophthalmos in several species of animals following the administration of various TSH preparations (1). To our knowledge there are no published reports on the exophthalmic potency of TSH in man. It is the purpose of this report to show that the short-term administration of thyroid stimulating doses of certain TSH preparations does *not* cause exophthalmos in man.

Methods and procedure. In the course of clinical studies on the use of TSH injections as a means of differentiating pituitary from primary hypothyroidism in patients(2), serial quantitative measurements of the amount of protrusion of the eyeballs of these patients were obtained before, during and after the administration of the hormone. Exact measurement of proptosis was accomplished with the aid of the Hertel exophthalmometer. One of us (B.S.) obtained all of the readings. Measurement of proptosis was made daily during the administration of TSH, and each daily measurement represented the average of 3 readings with the exophthalmometer.

In this study, exophthalmometer readings were obtained in 3 groups of patients. The *control* group consisted of 14 untreated patients in whom 2 daily consecutive exophthalmometer measurements were made. The second group consisted of 5 patients who had *intact thyroid glands* capable of functioning; all 5 of these patients had a marked increase in radioactive iodine uptakes by their thyroid glands following the administration of TSH (Table I). Two of these patients had pituitary hypothyroidism and the other 3 patients were euthyroid. The third group consisted of 8 *athyreotic* hypothyroid patients; these patients were proved to be athyreotic by failure of their thyroid glands to be stimulated by TSH injections (Table I). The 13 patients in the last 2 groups received daily doses of 25 or 30 mg of TSH by intramuscular injection for from 3 to 22 days. This dose of TSH was found to be thyroid-stimulating in those patients with intact thyroid glands. Nine patients received a Parke-Davis TSH preparation, Lot No. 50P5; 3 patients received a preparation of Armour TSH, Lot No. K26002; and one patient was given Armour TSH, Lot No. K39405.†

Results and discussion. Two daily consecutive exophthalmometer measurements in the 14 *untreated control* patients resulted in a mean change in readings of —0.014 ± 0.096 mm ($p = 0.88$). The administration of TSH to the group of 5 patients with *intact thyroid glands* and to the group of 8 *athyreotic* patients resulted in mean changes in exophthalmometer reading of —0.119 ± 0.102 mm ($p = 0.24$) and +0.033 ± 0.075 mm ($p = 0.67$), respectively. These data show that the administration of TSH to patients both with or without intact thyroid glands caused no greater changes in exophthalmometer readings than those observed upon 2 consecutive daily readings in the untreated or control group. In short, thyroid-stimulating doses of TSH did *not* produce exophthalmos in our patients under the conditions of this experi-

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TABLE I. Effect of TSH on Thyroid I^{131} Uptake.

Thyroid intact				Athyreotic			
	Thyroid I^{131} uptake				Thyroid I^{131} uptake		
No. of days of TSH	Before TSH	After TSH	Incr. I^{131} uptake	No. of days of TSH	Before TSH	After TSH	Incr. I^{131} uptake
3	7%	65%	+58%	12	4%	3%	—1%
5	5	69	+64	3	0	0	0
5	21	44	+23	5	3	2	—1
3	19	42	+23	5	2	3	+1
22	1	14	+13	5	17	23	+6
				3	10	15	+5
				5	3	5	+2
				11	0	1	+1
Mean	10.6	46.8	+36.2	Mean	4.9	6.5	+1.6

ment and with the particular TSH preparations used.

These results are contrary to previous animal observations in which anterior pituitary preparations with thyrotrophic activity induced exophthalmos in the guinea pig, rabbit, and a fish (Fundulus), within a time period of 24 to 72 hours. Possible explanations for this discrepancy are: 1) the dose of TSH given to our patients was too small; 2) TSH was not administered to our patients for a long enough period of time; 3) there is a species difference in the ability of the orbital tissues to respond to TSH; 4) there may be differing degrees of exophthalmic potency of various TSH preparations; and consequently, 5) exophthalmos may be caused by a pituitary exophthalmic factor which is *not* TSH but which is closely associated with it when whole pituitaries are fractionated by chemical means. We have not been able to test the first 3 points as yet, but plan to do so in the future. Concerning the last 2 points, Dobyns(3) has shown that there may be differing degrees of exophthalmic potency of various TSH preparations, and studies by Jefferies(4) on the inactivation of TSH with iodine suggest that the pituitary exophthalmic factor may not be identical to thyrothrophic hormone.

Summary. The parenteral administration of thyroid stimulating doses of 3 thyrothrophic hormone preparations in 13 patients both with and without thyroid glands for from 3 to 22 days failed to cause any statistically significant increase in proptosis. Under the conditions of this experiment, the short-term administration of TSH did not cause exophthalmos in man.

We are indebted to Dr. Frederick Moore, University of Southern California, School of Medicine, for the statistical analysis of the data.

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Response of Metacorticoid Hypertension to Bistrium, Apresoline, Veriloid and Serpentina. (20554)

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Metacorticoid, or post-DCA, hypertension is a self-sustaining cardiovascular disease attained in rats approximately 2 months after the implantation of a desoxycorticosterone acetate (DCA) pellet and the substitution of saline for drinking water(1-4). In many respects this type of hypertension appears similar to essential hypertension in man(1,5); as a consequence, it should prove useful for screening compounds of possible value in the treatment of humans. To test this hypothesis, we have investigated the activity of several agents known to have some effect in essential hypertension: Bistrium (hexamethonium iodide, Squibb), Apresoline (1-hydrazinophthalazine, Ciba), Veriloid (a purified alkaloidal fraction of *Veratrum viride*, Riker), and Serpentina (powdered roots of *Rauwolfia serpentina*, Penick).

Materials and methods. Acute experiments: Groups of male Sprague-Dawley rats were used which had received a single 20 mg pellet of DCA 3 to 6 months previously, and had been maintained on 0.86% NaCl solution and Rockland Rat Diet *ad libitum*. The systolic blood pressures of treated rats were estimated with a photoelectric tensometer(6) immediately before and 2, 4½, 6½ and 25 hours after treatment, and were compared to simultaneous readings from saline-injected controls. The mean blood pressure of over 400 normal rats has been determined by the tensometer method as 120 mm Hg, with a standard deviation of 10. Pressure readings on individual rats were made in ignorance of the treatment the rat had received and of the previous pressure reading. Each test was completed by one person on the same tensometer in a darkened room at constant temperature. The rats were trained for at least 3 months before testing, and were deprived of food and saline from the first pressure reading until after the 6½-hour reading. Saline solutions of Bistrium, Apresoline, and Veriloid were administered by stomach tube or subcutaneous injection at several dose levels (Table I). Although the *t*-tests for significance(7) utilized data from single control experiments, these data are averaged in Table I for brevity. Chronic experiment: Wilkins and Judson(8) reported that the response of hypertensive patients to *Rauwolfia serpentina* usually is not seen until after 3 to 6 days of administration. Accordingly, for 10 days 6 hypertensive rats (mean weight = 440 g) were offered *ad libitum* ground Rockland Rat Diet containing 5% powdered *R. serpentina* roots. Six hypertensive controls of similar body weight received Rat Diet only; both groups were offered 0.86% NaCl solution to drink. Blood pressures were taken as above, immediately before, and after 3, 5, 7, and 10 days of treatment (Table I).

Results and discussion. Single injections of Bistrium, Apresoline, and Veriloid significantly reduced blood pressures below simultaneous control readings, although when normotension was achieved, it was not generally maintained over the 25-hour period. On a dosage basis, Veriloid appeared to be the most active of the 3 compounds tested acutely. On the other hand, Bistrium and Apresoline, in the doses employed, seemed to have a greater duration of effect.

Serpentina was effective when administered in the food over a several day period. Assuming a food consumption of 80 g/kg/day (unpublished data), these rats were receiving approximately 4 g of root/kg/day.

Metacorticoid hypertension is not ameliorated by removal of the adrenals, thyroid, parathyroids or gonads, nor by drastic dietary restriction of Na or K(1). Hypophysectomy (2) and nephrectomy(3) abolish the hypertension, while Dibenamine(1) and certain 11,17 derivatives of 4-androsten-3-one(9) lower the blood pressure temporarily. The

TABLE I. Therapy of Metacorticoid Hypertension.

Treatment	Dose, mg/kg	Route*	No. rats	Mean blood pressure of treated rats, mm Hg: Before inj.	2 hr†	4½ hr	6½ hr	25 hr
Apresoline	5	SC	3	193	175	179	183	180‡
	10	"	8	192	154‡	157	167	180
	25	"	4	189	162§	150§	146§	152§
	200	ST	3	189	170‡	176	198	—
Control			20	192	192	187	189	191
Bistrium	32	SC	8	188	185	157§	148§	166§
	40	"	8	189	145§	148§	158§	181‡
	20	ST	4	189	185	184	181	192
	320	"	4	198	138§	181	167	156§
Control			24	192	190	188	190	193
Veriloid	.05	SC	4	200	203	188	190	194
	.10	"	4	200	190‡	186§	178§	188
Control			8	200	198	199	202	198
				Before inj.	3 days†	5 days	7 days	10 days
Serpentina	5% in food		6	177	142§	146‡	139‡	144‡
Control			6	178	183	168	166	170

* SC = subcut.; ST = by stomach tube.
† Indicates hr or days after inj.
‡ P <.05 that treat mean = control mean.
§ P <.01 that treat mean = control mean.

present data do not contradict the thesis that metacorticoid hypertension closely resembles those cases of essential hypertension in which the maintenance of elevated blood pressure does not involve adrenocortical participation(1).

Summary. Single injections of Bistrium, Apresoline, and Veriloid induced significant temporary reductions in the blood pressure of rats with metacorticoid (post-DCA) hypertension. Similar rats treated chronically with Serpentina root exhibited a sustained depression of blood pressure.

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Some Hormonal Effects on Phosphorylation in the Liver of Rats.* (20555)

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The ratio of phospholipide and nucleoprotein synthesis in the liver of rats appears to be rather constant in animals maintained on various experimental diets, irrespective of the fat or protein content(1). There appears to be a constant relationship of the synthesis of phospholipides to that of the nucleoproteins, whether or not the liver is fatty, fibrosed or normal(1). This constant rate of synthesis of liver phospholipides appears changed very little by dietary intake, but may be influenced by metabolic requirements of the body. The thyroid gland plays a definite role in phospholipide metabolism(2). In experimental animals, thyroxine increases the rate of synthesis in the liver which reflects a similar increase in the plasma(3). Thiouracil and thiourea diminish the phospholipide turnover(3,4). The growth hormone produces an increase in the turnover of liver phospholipides in normal or in hypophysectomized rats(5). In view of the above findings, experiments were undertaken to ascertain the effects of various hormones on phospholipide and nucleoprotein synthesis in the liver.

Methods. Male, albino rats (100-110 g) were divided into 3 groups, with a control for each group maintained on Diet 29(6). *Group 1.* A total of 37 animals received Diet 29(6) for 3 weeks. The diet for 16 of these was supplemented with 1% thiourea. *Group 2.* Eleven animals were maintained on Diet 29(6), fasted 24 hours and received an intraperitoneal injection of 5 mg of "beef thyrotropin" in saline (fat mobilization fraction of the pituitary)† and 13 controls received physiological saline. *Group 3.* Four animals were maintained on Diet 29(6) and received daily intraperitoneal injections of 0.20 mg of pure growth hormone for 10 days and 3 controls received physiological saline. Radioactive phosphorus was administered intraperitoneally as inorganic phosphate, containing 2-4 μc of P^{32} and after 6 hours the animals were killed by decapitation. The liver was removed, rapidly weighed and, in most cases, divided into equal portions for analyses. The acid-soluble phosphorus was removed by extracting with 10% trichloroacetic acid (TCA) containing 0.4 M $MgCl_2$(8). The lipides were extracted from the TCA insoluble residue with alcohol and alcohol-ether and purified with chloroform(9). On aliquots of the chloroform solution, the weight of the lipides, the radioactivity(9), and phosphorus(10) were determined. After the solvent extraction (TCA, alcohol-ether), the liver residue was digested for 3 hours at 90°C in one normal NaOH to determine total protein-bound phosphorus (11). This fraction contained both the nucleoprotein and phosphoprotein(11,12). Phosphoprotein is only a minor component of animal tissue(12). On aliquots, the total phosphorus(10), nitrogen(13), and radioactivity were determined. From these results and the radioactivity measurements, the relative specific activities‡ of the phospholipides and nucleoproteins, and the ratio of phospholipide phosphorus to nitrogen were calculated.

Under the conditions of our experiment, an increase in the formation of phospholipides and nucleoproteins may reasonably be

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† This fat mobilization fraction was assayed using the 7 hr mouse method of Payne(7). Twelve mice receiving saline, 0.125 mg and 0.500 mg of beef thyrotropin showed respectively 4.6, 5.6 and 11.8% of fat in the liver. (Courtesy of Dr. S. L. Steelman, Armour Laboratories, Chicago).

‡ The relative specific activity is the ratio of the specific activities of the phospholipide P or nucleoprotein P to that of the acid-soluble P.

TABLE I. Effect of Thiourea, Thyrotropin (Pituitary Fat Mobilization Factor) and Growth Hormone on the Phospholipide and "Nucleoprotein" Synthesis in the Liver of Rats.

Exp. condition		No. of Samples	Total lipides, g	R.S.A.:* Phospholipide P (Per g of fat free tissue)		R.S.A.:* Nucleoprotein P	Phospholipide P / Nitrogen	R.S.A.* phospholipide P / R.S.A. nucleoprotein P
Thiourea								
Controls		22		.147 ±.024	—	.062 ±.013	.037 ±.004	2.454 ±.519
Thiourea		31		.090 ±.019	—	.051 ±.010	.034 ±.007	1.764 ±.276
Difference between means	*t*			9.605	—	3.549	1.764	6.269
	P			P <.01		P <.01	P >.05	P <.01
Thyrotropin								
Controls		25	.55 ±.10	.212 ±.020	.038 ±.006	.073 ±.012	.033 ±.003	2.954 ±.402
Thyrotropin		22	.83 ±.20	.205 ±.028	.036 ±.006	.084 ±.017	.036 ±.003	2.484 ±.289
Difference between means	*t*		6.01	.962	1.148	2.602	3.440	4.551
	P		P <.01	P >.05	P >.05	P <.05	P <.01	P <.01
Growth hormone								
Controls		6	.58 ±.06	.126 ±.012	.022 ±.002	.060 ±.008	.031 ±.003	2.127 ±.202
Growth hormone		8	.56 ±.07	.165 ±.016	.027 ±.003	.075 ±.007	.027 ±.002	2.217 ±.158
Difference between means	*t*		.58	4.824	4.142	3.747	3.314	.938
	P		P >.05	P <.01	P <.01	P <.01	P <.01	P >.05

* Relative specific activity.

Figures preceded by the ± sign indicate stand. dev. *t* is the test of significance applied to difference between the means. P is probability for chance occurrence of this difference.

assumed when higher values for the relative specific activities are found. Various factors, such as differences in the rates of absorption and in the equilibrium between intra- and extra-cellular specific activities of the inorganic phosphorus, may conceivably produce changes in the specific activity of the inorganic fraction. Comparisons of the relative specific activities should provide a means of reducing these causes of error.

Results and discussion. The data obtained from the present investigation concerning some hormonal factors which influence phospholipide and nucleoprotein metabolism, phosphorus, and nitrogen distribution in the 3 groups of animals are reported in Table I. To evaluate the statistical significance of the results, the *t* test of significance(14) was applied to the difference between the means of the results from each group.

On feeding thiourea, statistically significant decreases between fed animals and controls were noted when the relative specific activities (ratio of specific activity of phospholipide P or nucleoprotein P, respectively, to specific activity of acid-soluble P) of phospholipide or nucleoprotein fractions of the 2 groups were compared. However, if the ratio of relative specific activity of the phospholipide to relative specific activity of nucleoprotein is compared in the thiourea-fed animals as against the controls, it turns out that the ratio is lower in the thiourea-fed animals. This would indicate that phospholipide synthesis in the liver of animals fed thiourea, as evidenced by the P^{32} incorporation, is inhibited more than is nucleoprotein synthesis. If the synthesis of both fractions were inhibited equally, although P^{32} incorporation in the experimental animals would be reduced, the ratios of relative specific activity of phospholipides to relative specific activity of nucleoproteins would be the same in the 2 groups of animals.

It is of interest that this ratio of synthesis of phospholipides to nucleoproteins is constant in the liver, irrespective of dietary fat or protein content and is not influenced by the presence or absence of fat in the liver(1). Campbell and Kosterlitz(15) have demonstrated in the rat that the ratio of liver protein nitrogen to phospholipides remains approximately constant on various levels of dietary proteins. In the present studies, it has been found that with administration of the pituitary fat mobilization hormone (beef thyrotropin) there was a 51% increase of mobilization of the fat in the liver in 6 hours. This increase in total lipides was statistically significant ($P < 0.01$). However, there was no change in the phospholipide synthesis. Pituitary fat mobilization hormone apparently does effect nucleoprotein synthesis, for there was an increase in the relative specific activities of the nucleoproteins.

The growth hormone, as shown in Table I, fails to affect the total lipide concentration in the liver, but stimulates both phospholipide and nucleoprotein synthesis ($P < 0.01$). This effect of growth hormone on lipide phosphorylation is in agreement with that of Geschwind *et al.*(5).

Summary. 1. Thiourea diminished phospholipide and nucleoprotein synthesis in the liver of rats. 2. The fat mobilization fraction of the pituitary gave a 51% increase in total lipides in the liver of rats in 6 hours. However, there was no change in the synthesis of phospholipides, but a statistical increase in the synthesis of nucleoproteins. 3. The Growth Hormone increased both phospholipide and nucleoprotein synthesis in the liver of rats.

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Further Studies of Metabolic Removal of Alkyl Groups from Nitrogen in Barbituric Acid Derivatives.* (20556)

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In an earlier study(1) of the metabolic fate of N-substituted derivatives of barbital, it was found that when N-methyl barbital or N-ethyl barbital is administered to dogs, a considerable amount of barbital is excreted in the urine. A trace of pure barbital was recovered from the urine of one dog receiving N-*n*-propyl barbital; from another one none could be isolated. The isopropyl, allyl, *n*-butyl, and phenyl derivatives of barbital

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were not converted to barbital in sufficient amounts to allow isolation. By means of an analytical method for the determination of the methylated and demethylated compounds in plasma, a quantitative study of the conversion of N-methyl barbital (metharbital, Gemonil) to barbital has already been carried out(2). In the present study methods have been developed for the determination in plasma of N-ethyl or N-*n*-propyl barbital in the presence of barbital. These methods have been applied to an investigation of the physiological disposition of those 2 derivatives of barbital. An elucidation of the difference between the fates of the propyl derivative and of the 2 lower homologues was considered of particular theoretical interest. Also included in this report is a study of the rates at which the 2 methyl groups are removed from N,N′-dimethyl barbital. The conversion of this compound to barbital had previously been demonstrated (3), and the method for determination of monomethyl barbital and barbital in plasma (2) has now been used to follow the course of the double demethylation.

Materials. The N-ethyl, N-*n*-propyl and N,N′-dimethyl derivatives of barbital were synthesized by Dr. Milton T. Bush of Vanderbilt University(1,3). *Administration of drugs.* N-ethyl barbital and N-propyl barbital were given to dogs intravenously as freshly prepared aqueous solutions containing 1.1 equivalents of NaOH. N,N′-dimethyl barbital was injected intraperitoneally as a solution of 8 mg per ml in saline. *Determination of N-methyl barbital and barbital in plasma.* The method previously described was used(2). Unchanged N,N′-dimethyl barbital, not being ionizable, is not extracted from ether into the carbonate buffer. It is consequently not determined and does not interfere. *Determination of N-ethyl barbital and barbital in plasma.* In a glass stoppered centrifuge tube are placed 4 ml of oxalated plasma, 1 ml of buffer, pH 6 (0.86 moles KH_2PO_4 + 0.14 moles K_2HPO_4 per l), and 25 ml of ethyl ether previously purified by distillation and washing with solutions of NaOH and HCl and finally with water. After shaking and centrifugation, 20 ml of the ether phase is transferred to another tube and shaken with 4 ml of 0.2 *N* NaOH saturated with ether. After centrifugation the lower phase is withdrawn and shaken with 2 ml of ether. The tube is centrifuged and the ether discarded. To 3 ml of the aqueous phase is added 3 ml of 0.2 *M* H_3BO_3. The resulting pH is about 10, and both compounds to be determined are present essentially completely in the form of monopolar anions. The optical density is measured at 241, 250, and 260 mμ with a Beckman ultraviolet spectrophotometer. A mixture of equal parts of the NaOH and H_3BO_3 solutions is used as the blank. At 241 and at 250 mμ the absorption of N-ethyl barbital is equal, and it is from the difference between the densities at these 2 wavelengths that the concentration of barbital is calculated. The concentration of N-ethyl barbital is derived from the difference between the density at 250 mμ and that at 260 mμ. From the observed densities at these 2 wavelengths are subtracted the corresponding densities that would be contributed by the concentration of barbital found by the first calculation. The difference between the corrected density at 250 mμ and the corrected density at 260 mμ is the value from which the concentration of N-ethyl barbital is calculated. Errors due to interference from normal constituents of plasma have not exceeded values corresponding to concentrations of 2 mg per l of either drug. *Determination of N-propyl barbital and barbital in plasma.* The method is identical to that described for the ethyl compound except that a measurement at 242 mμ is substituted for that at 241 mμ.

Results and discussion. Following intravenous injection of N-ethyl barbital and N-propyl barbital in dogs, the resulting concentrations of unchanged drugs and dealkylated products are shown in Fig. 1 and 2, respectively. In both experiments there is an initial rapid drop in the concentration of unchanged drug, this being doubtless due to passage of the drug from blood into tissues rather than to actual loss from the body by chemical destruction or excretion. After this phase the decline in concentration is slow. The pattern of falling plasma concentration of both of these drugs resembles closely that previously found for N-methyl barbital(2).

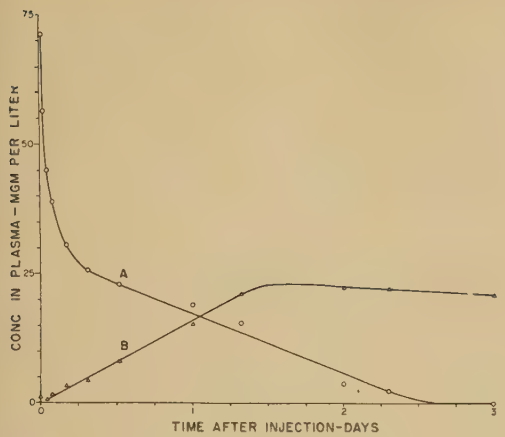

FIG. 1. Curve A (points designated by circles) shows the concentrations of unchanged drug and Curve B (points designated by triangles) the concentrations of barbital found in plasma following the intravenous inj. of 3×10^{-4} moles (64 mg)/kg of N-ethyl barbital in a 12 kg female dog.

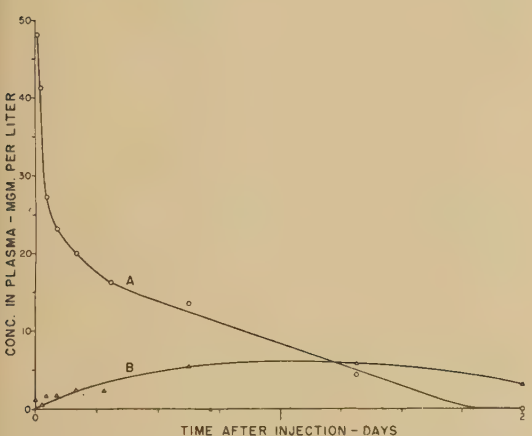

FIG. 2. Curve A (points designated by circles) shows the concentrations of unchanged drug and Curve B (points designated by triangles) the concentrations of barbital found in plasma following the intravenous inj. of 2.5×10^{-4} moles (57 mg) per kg of N-propyl barbital in a 12 kg female dog.

All 3 of these alkyl derivatives of barbital disappear at rates that are of the same order of magnitude.

As unchanged N-ethyl barbital disappears from the plasma, barbital appears and increases slowly in concentration for more than a day. Comparison with the corresponding experiment in which the same molar dose of N-methyl barbital was given to another dog (2) shows that barbital was produced somewhat more rapidly from the methyl than from the ethyl derivative but that the rates at which these 2 alkyl groups are removed from nitrogen are of the same order of magnitude.

Calculation of the yield of barbital by the procedure outlined in the earlier study(2) indicates that approximately 60% of the N-ethyl barbital that has disappeared after 2 days can be accounted for as barbital produced. While this estimate is necessarily rough, it does corroborate the conclusion(1), based on isolation of barbital from urine, that N-ethyl barbital is dealkylated to a large extent. If allowance is made for losses, the amounts isolated from urine are consistent with a yield of the order of that estimated here.

The metabolic fate of the N-propyl derivative of barbital is in striking contrast to that of the 2 lower homologues in that much less barbital is produced. In the experiment of Fig. 2, the highest concentration of barbital found is less than 4 times the normal blank. The measurements are consequently subject to a large proportional error, but do suffice to indicate that not more than about 10% of the N-propyl barbital was dealkylated. This experiment is thus corroborative of the earlier discovery(1) that N-propyl barbital does not give rise to significant amounts of barbital in the urine. It was suggested(1) that failure of N-propyl and some of the other N-substituted barbitals to be converted to any great extent to barbital might be due to their destruction by more rapid reactions rather than to any inherent incapacity of the body to remove the substituent groups from nitrogen. The brief duration of action of a single intravenous dose of N-propyl barbital was at that time taken as evidence of rapid chemical destruction. It is now known that the transient character of the effects seen after intravenous injection of this drug (and also other N-substituted barbituric acids and thiobarbituric acids) can be attributed to physical redistribution of the drug in the body rather than to chemical inactivation. It is evident in the experiment of Fig. 2 that N-propyl barbital persists in the body for a long time and that the low production of barbital cannot be due to rapid removal of the parent compound by other processes. Either the propyl group is less susceptible to enzymatic attack than the

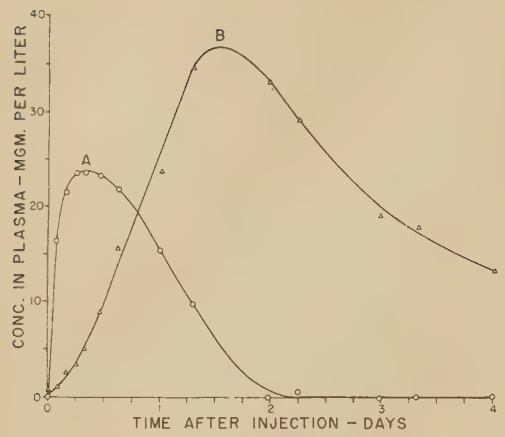

FIG. 3. Curve A (points designated by circles) shows the concentrations of N-methyl barbital and Curve B (points designated by triangles) the concentrations of barbital found in plasma following the intraperitoneal inj. of 3×10^{-4} moles (64 mg) per kg of N,N′-dimethylbarbital in a 12 kg female dog. Unchanged drug was not determined.

methyl and ethyl groups, or the initial product of enzymatic action is one in which there is less tendency toward rupture of the nitrogen-carbon bond.

N,N′-dimethyl barbital was injected intraperitoneally in a dog and the concentrations of the singly and doubly demethylated products in plasma measured. The results are shown in Fig. 3. The monomethyl compound appears at a relatively rapid rate. The final product of demethylation, barbital, appears more slowly and is increasing in concentration long after its immediate precursor has passed its maximum concentration. If it be assumed that the volumes of distribution are the same as found in an earlier experiment(2), it can be calculated that the initial rate at which dimethyl barbital is converted to monomethyl barbital is several times that at which the latter compound is converted to barbital. It might be expected that the probability of a methyl group being lost within a given period of time would be higher for a molecule containing 2 identical methyl groups than for a molecule containing only one. This factor alone would not, however, suffice to explain the high rate at which the first methyl group is removed from N,N′-dimethyl barbital.

Summary. Analytical methods are described for the simultaneous determination in plasma of N-ethyl barbital and barbital or N-propyl barbital and barbital. These methods have been used to study the metabolic fate in the dog of the N-ethyl and N-propyl derivatives of barbital. Both of these compounds disappear slowly from the plasma. N-ethyl barbital is converted in considerable part to barbital, the concentration of that substance in plasma increasing for more than a day. N-propyl barbital is converted to barbital only to a very small extent. By means of methods previously developed for the simultaneous determination of N-methyl barbital and barbital in plasma, the fate of N,N′-dimethyl barbital has been studied. Both methyl groups are removed from this compound, the first much more rapidly than the second.

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Concentration of Ascorbic Acid in Human Adrenal Cortex Before and After ACTH Administration.* (20557)

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(Introduced by Philip E. Smith.)

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It has been demonstrated that there is considerable species difference in adrenal ascorbic acid content, the concentration in the rat adrenal being about 3 times that found in the guinea pig(1). A considerable variation has also been reported within the same species, a range of 30 to 154 mg % having been found in the dog by Peters and Martin(2), who also found that there was little post-mortem change in the adrenal ascorbic acid content of the dog up to 6 hours post-mortem at "Morgue temperature" (12°C). This observation appears to validate the findings of Yavorsky, Almaden, and King(3) on human adrenals from autopsy material. These investigators found an average adrenal ascorbic acid level of 55 mg %. Since the work of Sayers *et al.*(4-6) it has become apparent that much of the wide variation in ascorbic acid content of the adrenal is the result of depletion consequent to the action of adrenocorticotropic hormone, and that the lowering of adrenal ascorbic acid may be brought about through the action on the pituitary of noxious stimuli, such as cold, "shock", injection of toxins, hemorrhage, etc. The fall of adrenal ascorbic acid following stimulation does not occur in all species, (the chick(7) and the quail(8) failing to respond). Agate, Hudson, and Podberezec(9) have reported the fall of cortical ascorbic acid following ACTH in the human.

The opportunity was recently presented to gather some data on human adrenal ascorbic acid levels in surgically removed glands with only a relatively short time elapsed between surgical excision and determination of ascorbic acid content. It was also possible to obtain glands from patients who had been previously treated with ACTH or cortisone or both.

Materials and methods. The data presented here were secured from the surgically excised adrenal glands of 22 patients of both sexes, all of whom had metastases, in most cases from a prostatic or a mammary carcinoma. Only the data on the ascorbic acid content of the adrenal cortex are presented here. In one group (11 patients) bilateral adrenalectomy was performed at one operation. All patients in this group were treated for 24 hours pre-operatively with 350 mg of cortisone in 5 divided doses. The right adrenal was removed and in 8 cases immediately after excision 25 U.S.P. units of ACTH were administered intravenously. The second adrenal (the left) was removed after a variable interval of time ranging from 40 to 180 minutes. In 3 cases in this group *no* ACTH was administered and the second gland was removed after an interval of 75 to 90 minutes. In a second group the adrenalectomy was done in 2 stages; some patients were treated with cortisone preoperatively and some were not. Six patients received 25 U.S.P. units of ACTH intravenously at timed intervals ranging from 40 to 300 minutes prior to removal of the first gland. After a period of 14 to 30 days the second adrenal was removed from 8 of these patients after treatment with 350 mg of cortisone divided into 5 doses during 24 hours. No ACTH was administered prior to the removal of the second adrenal. In 3 patients, in this group, one ACTH treated and 2 treated with neither cortisone nor ACTH, death intervened before the scheduled time for the removal of the second gland. It should be mentioned that no clinical or laboratory evidence of adrenal insufficiency was found

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in any of these patients after the removal of a single adrenal. Immediately after excision, the gland was cut into slices about 2-5 mm in thickness. Some slices were fixed for routine pathological study. Several slices from each gland were used for ascorbic acid determination, slices adjacent to each of these being fixed for histological study. An outline was traced of each slice to be used for chemical determination. Small segments were then cut out ranging from 20-60 mg in weight and the outline of the removed segment was indicated on the section outline. By comparison of this map of the material used for ascorbic determinations with suitably stained microscopic sections it was possible to ascertain what regions of the gland, medulla, cortex, or even various zones of the cortex, were included in the determination. Only determinations on cortex are included, data from segments having medulary tissue, excessive quantities of connective tissue, or large blood vessels being excluded. The segment of gland used for ascorbic acid determination was weighed immediately and ground with acid washed Berkshire sand in 2 cc of 6% trichloroacetic acid. The total ascorbic acid was then determined by the method of Roe and Kuether. Determinations were done on at least 6 segments from each gland, usually on 10 or 12.

Results. Fig. 1 shows the mean cortical ascorbic acid content of all the glands in the entire series. The first column represents the mean plus or minus the standard error in mg of ascorbic acid per 100 g of cortex (135 ± 5) for males and females combined who were treated prior to operation with cortisone but *no* ACTH. The number in each column represents the number of glands included. The second column is the mean (129 ± 9.3) for those glands which received neither cortisone nor ACTH preoperatively. The third and fourth columns represent grouping according to sex of those glands which received no ACTH (131 ± 8 and 139 ± 5). The majority received cortisone but the 3 cases which received no cortisone are included. There appears to be no significant difference between the first 4 columns. The last 2 columns represent the cortical ascorbic acid of all glands in the series following ACTH treatment. The mean for the ACTH-treated males is 97.3 ± 9 mg % as compared with a value for the males with no ACTH of 131 ± 8. The mean for the ACTH-treated females is 98 ± 10, as compared with 139 ± 5 for the females with no ACTH.

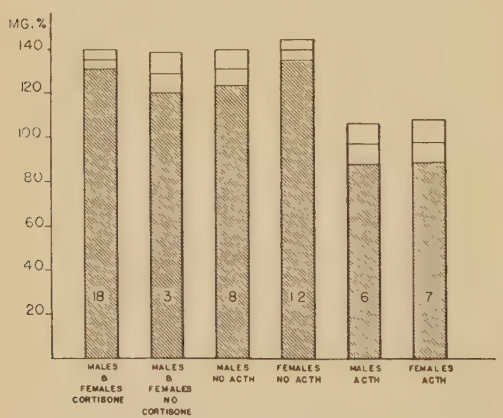

FIG. 1. Heights of the bars indicate cortical ascorbic acid content in mg/100 g fresh tissue. Unshaded area at top of bar represents range of plus and minus stand. error of mean, the mean being represented by the horizontal line in the center of this area. For further details see text.

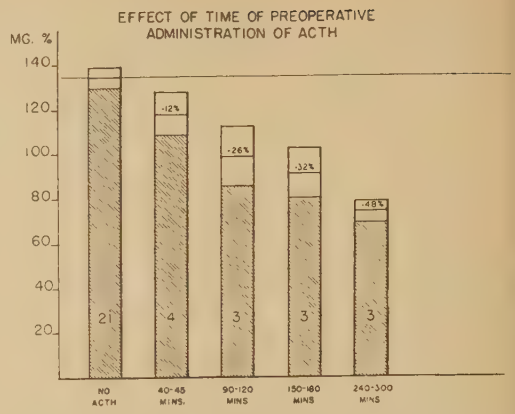

FIG. 2. Vertical coordinate is same as for Fig. 1. Numbers toward top of each bar represent % departure of mean from the mean of glands from patients not treated with ACTH. For further details see text.

Fig. 2 represents the effect of time of administration of 25 U.S.P. units of ACTH on the ascorbic acid content of the cortex. All figures are for males and females combined. The first is the mean of 134 ± 4.4 mg % for all untreated glands. Subsequent values (40-45 minutes, 90-120 minutes, etc.) show a

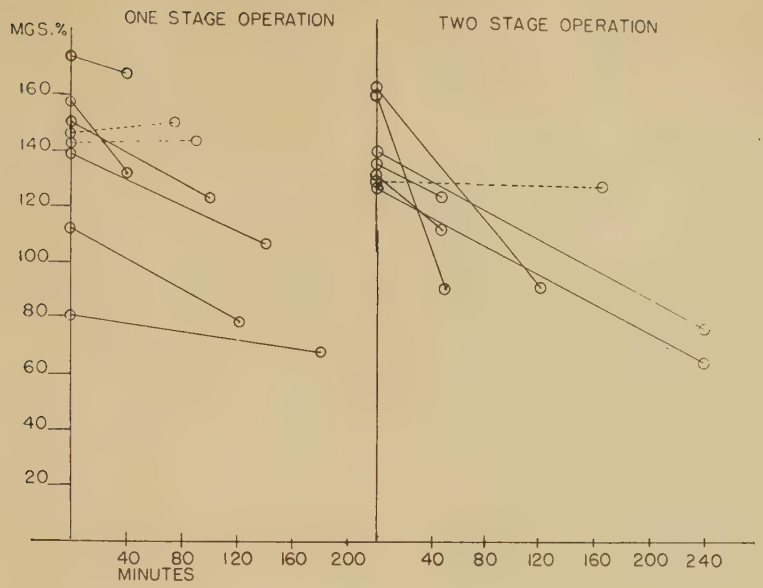

FIG. 3. See text.

steady decline to 91 ± 11 mg % at 150-180 minutes after ACTH. The last column at 240-300 minutes (74 ± 4.8 mg %) is complicated by the fact that 2 of these patients received an additional 50 U.S.P. units of ACTH during the operation making a total dose of 75 units.

Fig. 3 shows some individual cases. The left half of the chart shows cases of the first group which were bilaterally adrenalectomized in one stage. The points at the left represent the cortical ascorbic acid value of the first adrenal which was removed prior to ACTH administration. Each point is connected by a solid line with the ascorbic acid value for the second adrenal removed after 25 U.S.P. units of ACTH, the horizontal axis representing the time between administration and excision of the gland. The 2 dotted lines represent 2 cases in which no ACTH was given. In these cases the time indicated is that elapsed between the removal of the first and second glands. On the right half of Fig. 3 are shown values for the 2-stage operation. In these cases the values at the left represent the second gland which was removed with no ACTH treatment. The value for the first gland removed 2 weeks to a month earlier is shown toward the right, the time represented being the time elapsed between the administration of 25 U.S.P. units of ACTH and excision of the gland. The dotted line shows values for a case which received no ACTH prior to the removal of either gland and the time indicated is the duration of surgery up to the time of excision of the first gland.

Discussion. The primary purpose of cortisone administration prior to adrenalectomy was to protect the patient from the effects of sudden cortical steroid deficiency. However, we felt, in view of the finding of adrenal atrophy in humans following prolonged cortisone administration(10), that cortisone in the dosage used probably inhibited the release of endogenous ACTH from the patient's hypophysis during the course of the operation.

This appears to be substantiated by 2 cases at a one-stage operation and one case at a 2-stage operation, which received cortisone but no ACTH (Fig. 3, dotted lines).

The usual dosage of ACTH used in this series of humans ranged from 300-550 milliunits per kg of body weight. The mean fall in cortical ascorbic was only 12% at 40 to 45 minutes, but reached 26% at 90-120 minutes and 32% at 150 to 180 minutes. Under the conditions in our laboratory, using 100 g male rats at 24 hours after hypophysectomy a 30% depletion is obtained in adrenal ascorbic acid at 60 minutes after intravenous injection of

50 to 70 milli-units per k body weight. As demonstrated by Sayers(4), the ascorbic acid response in the rat reaches a maximum at about 20-30 minutes after ACTH injection.

In evaluating the data in this series it is important to bear in mind that all the adrenals included in this series were from patients with relatively advanced carcinomata and no comparable data are as yet at hand from human adrenals of noncancerous patients.

Summary. 1. The mean ascorbic acid content of the adrenal cortex of human cancer patients prior to ACTH treatment was found to be 131 ± 8 mg per 100 g tissue in the male and 139 ± 5 mg % in the female. 2. A 32% depletion in cortical ascorbic acid was found at 2 to 3 hours after intravenous injection of 25 U.S.P. units of ACTH. 3. The dosage of ACTH required to produce this depletion appears to be about 7 to 8 times that required per kg of body weight for the male hypophysectomized rat and the time elapsed between ACTH injection and maximal ascorbic depletion appears to be greater than 3 hours in the human.

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Effect of Continuous Injection of Epinephrine upon the Glycosuria of Partially Depancreatized Rats. (20558)

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In acute experiments on intact animals, epinephrine causes hyperglycemia, redistribution of glycogen stores and occasionally a mild glycosuria of short duration. The effects of the prolonged injection of epinephrine are less well studied. Epinephrine was administered continuously to normal and to partially depancreatized rats for periods of 3 and 4 weeks. Increased amounts of glucose were excreted during the injection of epinephrine.

Methods. Male rats of the Sprague-Dawley strain were maintained on Archer Dog Pellets until they were partially depancreatized at a weight of 275 to 280 g. Depancreatized rats and unoperated rats were thereafter force-fed a medium carbohydrate diet(1) by stomach tube each morning (8:30 a.m.) and afternoon (4:15 to 5:00 p.m.). Each 24-hour dose of epinephrine hydrochloride (Upjohn) was contained in 1 ml of physiological saline with 1 mg of ascorbic acid added as an antioxidant. The control rats received an equal amount of saline and ascorbic acid. Each rat was placed in a metabolism cage which restricted its activity so that the animal was unable to reverse its position. A 21-gauge needle, having a small barb attached to its shank to prevent its withdrawal, was placed subcutaneously. Sterile needles were used for replacement every 48 hours. The solutions were administered to 6 rats simultaneously by a continuous injection machine. Three of the 6 rats received epinephrine and 3 received control injections. The room temperature was 74° to 78°F. Urine was collected at the same hour (8:00 to 8:30 a.m.) each day for the quantitative determination of glucose(2).

Experiments and results. Exp. 1 (Fig. 1)

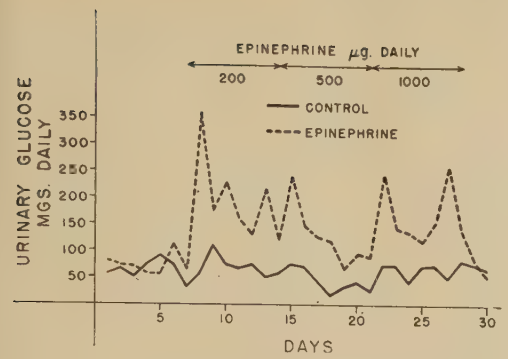

FIG. 1. Urinary glucose of normal rats with and without epinephrine. Avg for 6 rats/group.

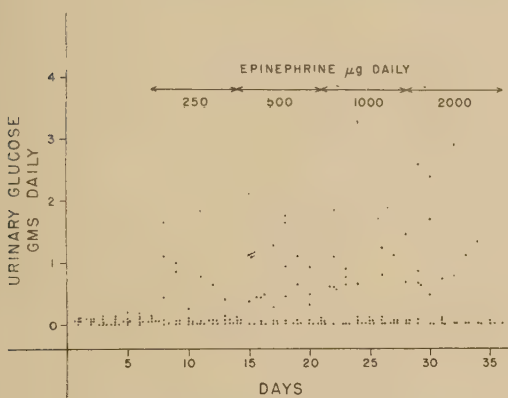

FIG. 2. Effect of epinephrine upon urinary glucose of 6 partially depancreatized rats without spontaneous glycosuria. Individual values.

involved 12 normal rats. Following a control period of 7 days, each of 6 rats received daily 200 μg of epinephrine for 7 days, 500 μg for 7 days and 1000 μg for 7 days, followed by a second control period for 7 days. The 6 control rats simultaneously received 1 ml of saline per rat daily for 21 days. All of the rats excreted small amounts of reducing substances during the control periods. During the injection of epinephrine there was a sharp but small rise in the excretion of reducing substances which tended to abate during the continued administration of that dose until the increase in dosage caused a small and temporary rise. When the injection of epinephrine was stopped, the excretion of reducing substances fell to control levels.

Exp. 2 (Fig. 2) involved 6 partially depancreatized rats which did not excrete more than 100 mg of reducing substances per rat per day during the control periods. Epinephrine was administered in doses per rat per day of 250, 500, 1000, and 2000 μg for 7 days per dose. Each rat excreted significant amounts of glucose on several days during the injection of epinephrine. The glycosuria tended to occur within 24 to 48 hours after the beginning of injections or upon an increase in dosage. Four of the rats died during the administration of 2000 μg of epinephrine daily.

Exp. 3 (Fig. 3) involved 18 mildly diabetic rats. Following an initial control period of 7 days, each of 9 rats received daily 200 μg of epinephrine for 7 days, 500 μg for 7 days and 1000 μg for 7 days. There followed a second control period of 7 days. The 9 control rats each received 1 ml of saline daily during the 21-day injection period. During the injection of 200 μg of epinephrine per rat per day there was a temporary increase in glycosuria, there was a slight rise when the dose was increased to 500 μg daily and a more striking but temporary rise when the dose was increased to 1000 μg daily. When the injections were stopped, the glycosuria decreased to values similar to those of the control animals which had shown a gradual decrease in glycosuria.

Discussion. In acute experiments epinephrine stimulates hepatic glycogenolysis. This is not the only effect of epinephrine upon carbohydrate metabolism for it inhibits glucose tolerance in the liverless rat(3), and there is other evidence(4) that it affects the peripheral distribution and utilization of carbohydrate. In partially depancreatized rats, epinephrine can cause the excretion of more glucose than

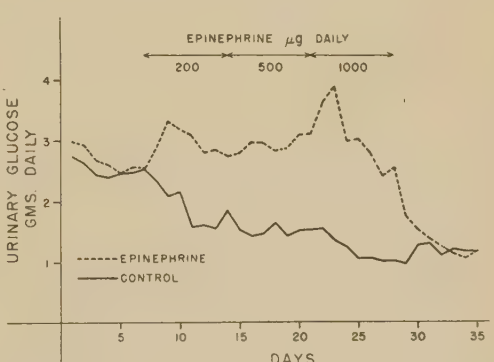

FIG. 3. Urinary glucose of mildly diabetic rats with and without epinephrine. Avg for 9 rats per group.

is present in liver at any one time. Rats without spontaneous glycosuria (Exp. 2, Fig. 2) excreted up to 3 g of glucose during a 24-hour period. If this temporary exacerbation of diabetes is due to an effect of epinephrine upon liver, it must be described in terms of changes in turn-over rather than as a single discharge of glucose from liver. The maximum glycosuria usually occurred on the 2nd day after beginning the continuous injection of epinephrine.

The possibility that epinephrine stimulates the adrenal cortices to secrete diabetogenic amounts of cortical hormones can be considered. Large doses of epinephrine can stimulate the adrenal cortices via the increased release of corticotropin in acute experiments(5), but there is no satisfactory evidence known to the authors that a true state of hypercorticalism can be induced by epinephrine.

The largest dose of epinephrine used in this study, 1000 μg per day, is near the maximum that can be tolerated by the rat. When the dose was doubled, as in Exp. 2, some of the rats died. In preliminary studies it was found that either a dose of 500 or 1000 μg of epinephrine per rat per day would cause death in some animals unless the animals were first adapted to a smaller dose.

Summary. Epinephrine was administered to normal and to partially depancreatized force-fed rats by continuous subcutaneous injection for periods of 3 and 4 weeks. Simultaneously the control animals were injected with saline. Increased amounts of glucose were excreted by both normal and partially depancreatized rats during the injection of epinephrine. The peak response occurred on the second day after starting the injection of epinephrine or increasing the dose. All of the animals showed adaptation to epinephrine so that the peak response was not sustained.

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Enzymatic Evidence for Intrinsic Oxytocic Activity of the Pressor-Antidiuretic Hormone. (20559)

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Until recently there has been uncertainty as to whether the oxytocic activity of vasopressin preparations was due to contamination of the preparations with oxytocin or to an intrinsic oxytocic activity of the pressor-antidiuretic hormone. Evidence for the latter explanation was afforded by the work of Popenoe, Pierce, du Vigneaud, and van Dyke (1). They found that the most highly purified arginine-vasopressin prepared in this laboratory possessed approximately 600 pressor units per mg by the arterial blood pressure method in cats and an oxytocic activity of 80-90 units per mg by the method of Coon(2), based on the lowering of the blood pressure of the fowl, and 30 units per mg by the rat uterus method.† On the other hand, a highly purified oxytocin preparation(3) possessing

* The authors wish to express their appreciation to the Lederle Laboratories Division, American Cyanamid Co., for a research grant which has aided greatly in this investigation, and to thank Parke, Davis and Co., and Armour and Co. for gifts of posterior pituitary material used in the preparation of the arginine- and lysine-vasopressin.

† Coon(2) found that when the pressor-oxytocic ratio of a pituitary solution was above 2.5 the oxytocic assay value obtained by the depressor method was higher than that obtained by the guinea pig uterine method.

an oxytocic potency of 500 units per mg had no effect on the arterial blood pressure of cats and less than 0.5 unit per mg of antidiuretic activity in the *diabetes insipidus* dog(1).

Analytical studies indicated that the vasopressin preparation mentioned could not have been contaminated with sufficient oxytocin to account for the oxytocic activity. Previously it had been shown that oxytocin was a polypeptide containing leucine, isoleucine, tyrosine, proline, glutamic acid, aspartic acid, glycine, cystine, and ammonia. Analysis of this vasopressin preparation by starch column chromatography(4) yielded phenylalanine, tyrosine, proline, glutamic acid, aspartic acid, glycine, arginine, cystine, and ammonia, but no detectable leucine or isoleucine. It was therefore concluded that vasopressin possessed intrinsic oxytocic activity. Additional support of this conclusion has come from zone electrophoresis studies reported by Taylor, du Vigneaud, and Kunkel(5). After the establishment of the isoelectric point and the mobility of oxytocin and vasopressin it became obvious that if purified oxytocin and vasopressin were mixed together, one ought to obtain 2 oxytocin peaks and one vasopressin peak with one of the oxytocin peaks coinciding with the pressor peak if the pressor material actually possessed oxytocic activity. The prediction was borne out by such a study. A third line of evidence for the intrinsic oxytocic activity of vasopressin is presented in this paper. The enzyme, trypsin, had been shown by Croxatto and Croxatto(6) and by preliminary studies on highly purified material in this laboratory‡ to destroy the pressor activity of vasopressin preparations but not the oxytocic activity of oxytocin preparations. A study of the effect of trypsin on the oxytocic and pressor activity of an arginine-vasopressin preparation free of leucine and isoleucine and of a lysine-vasopressin preparation was therefore undertaken. Control studies were made of the enzyme action on oxytocin.

In agreement with the earlier work, the oxytocic activity of oxytocin was not destroyed by trypsin but the pressor activity of vasopressin was destroyed. However, the present work shows that the oxytocic activity of arginine-vasopressin in contrast to the oxytocic activity of oxytocin is destroyed by trypsin. Thus additional, convincing proof that the oxytocic activity of vasopressin is not due to oxytocin and that vasopressin has intrinsic oxytocic activity is offered. The study of lysine-vasopressin gave parallel results. Undoubtedly the basic amino acids present in the vasopressin preparations make these hormones susceptible to an enzymatic attack to which oxytocin is resistant.

Experimental. Pressor and oxytocic activities reported in this study were based on assays standardized by reference to the U.S.P. Posterior Pituitary Standard. Oxytocic assays were performed according to the chicken depressor method of Coon(2). Pressor assays were obtained by measurements of the arterial blood pressure response of cats anesthetized with sodium phenobarbital. Arginine-vasopressin was prepared from bovine posterior powder by the method already described(7). It possessed 390 units per mg of pressor activity and 54 units per mg of oxytocic activity by the chicken depressor assay. The preparation was free of leucine and isoleucine.

Forty-five mg of the arginine-vasopressin preparation and 1.8 mg of trypsin§ were dissolved in 5 ml of water and adjusted to pH 7 with $NaHCO_3$. The mixture was incubated at 38°C for 5.5 hours. Assays before and after incubation showed that there was an almost complete loss of pressor and of oxytocic activity. It was apparent that the enzymatic degradation of the vasopressin preparation had resulted in a destruction of both pharmacological properties. Control studies of the effect of trypsin on oxytocin demonstrated that the oxytocic activity was not destroyed and thereby substantiated the above conclusion.

The effect of trypsin on lysine-vasopressin which had been prepared from hog glands(8) was also investigated. The initial activity of

‡ Taylor, S. P., and du Vigneaud, V., unpublished data.

§ Crystalline trypsin containing approximately 50% Mg SO_4 was obtained from the Worthington Biochemical Laboratory, Freehold, N. J.

the preparation was found to possess approximately 175 pressor units per mg, 30 oxytocic units per mg by the chicken depressor method, and 9 oxytocic units per mg by the rat uterus method. After the incubation with trypsin there was a destruction of the oxytocic as well as the pressor activities of the lysine-vasopressin.

Summary. 1. It has been shown that trypsin had no effect on the activity of oxytocin but destroyed both the pressor and oxytocic activities of arginine-vasopressin. These conclusions offer enzymatic evidence, in addition to the evidence already obtained from analytical and electrophoretic studies, for the inherent oxytocic activity of arginine-vasopressin. 2. It has also been demonstrated that a lysine-vasopressin preparation possessed oxytocic activity and that this activity was destroyed with the pressor activity by trypsin.

The authors wish to express their appreciation for the capable assistance of Miss Mary Anne De Witt and Miss Sylvia Kirsimagi with the biological assays.

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Effect of Alloxan on Isolated Liver of the Bull Frog.* (20560)

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The mechanism of the alloxan-induced initial fluctuations of the blood sugar is not yet clearly understood. Two alternate hypotheses have been offered to explain the alloxan hypoglycemia. A pancreatic origin seemed to be implied mainly by the observations that the hypoglycemia is a) preceded by histological evidence of Beta cell degeneration(1), and b) absent in animals who were previously depancreatized or made diabetic by alloxan or whose pancreatic blood supply is occluded temporarily while alloxan is injected intravenously (2,3). It appeared from this evidence that the transitory hypoglycemia was the result of endogenous insulin leaking from the disintegrating beta cells. This interpretation was challenged, however, when it was found that a) alloxan hypoglycemia could still be induced in freshly depancreatized dogs at least in some instances(4-6), and b) the insulin content of the pancreatic islets does not decrease until several hours after the onset of the hypoglycemic phase(6). Therefore, an extrapancreatic —probably hepatic-origin of the alloxan hypoglycemia was postulated(7,8). A direct effect of alloxan on the liver of the frog was demonstrated by Houssay and Gershman(9) in perfusion experiments where alloxan was found to inhibit glycogenolysis. Decrease of the spontaneous glycogenolysis was also shown by Kepinov(10) in the isolated and perfused rat liver. Weber(11) presented evidence that alloxan diabetic rabbits are able to store more glycogen in the liver after glucose feeding than normal animals. Other investigators using

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various approaches have failed, however, to confirm such direct hepatic action of alloxan (12-14). Recently we have carried out a series of experiments with perfusion of the isolated liver of the bull frog and have been able to demonstrate that alloxan significantly inhibits both the spontaneous and the adrenalin induced glycogenolysis.

Material and methods. The liver preparation for perfusion was set up according to the original description by Froehlich and Pollak (15) and the modification as to rate of flow and to pressure by Geiger and Loewi(16). Bull frogs—rana catesbeiana—of 200-250 g weight, were used and kept in hibernating condition at 4°C. The animals were killed by decapitation without use of an anesthetic. The liver was removed while preserving the abdominal vein and the venous sinuses; it was placed on a wire gauze plate on a tripod under which a small collecting beaker was placed. The perfusion system consisted of 2 funnels connected by rubber tubings with a 3-way stop cock, the third rubber tubing being mounted with a No. 26 needle. This needle was inserted into the abdominal vein and secured by ligation. The perfusate passed through this system and the liver with constant pressure so that 5-7 drops per minute were collected from the venous sinuses. Amphibian Ringer solution(17) (NaCl 0.66, KCl 0.015, $CaCl_2$ 0.015, Na HCO_3-Ph 7.8, aqu. dest. ad 100) and amphibian Ringer-Glucose solution which contained 200 mg% glucose were used as perfusates and placed into either of the 2 funnels. Where oxygenated solutions were employed saturation was maintained by bubbling O_2 through the perfusate in the funnel. When the effects of alloxan or adrenalin were studied, the material was injected directly into the rubber tubing near the abdominal vein. The preparation of the livers for perfusion was completed within 5 minutes. The perfusion experiments were carried out at room temperature over periods of 3 hours, during which time the livers appeared healthy and maintained almost normal color and consistency. The organs were weighed before and after the experiment; a gain of about 10% of the original wet weight was noted in most experiments, due to retention of perfusate.

TABLE I. Effect of Perfusion with Amphibious Ringer Solutions without Oxygen, with Oxygen and with the Addition of 200 mg % Glucose on Glycogenolysis of the Surviving Liver of the Bull Frog.

Perfusate	Time in min.																				
	10	20	30	40	50	60	70	80	90	100	110	120	130	140	150	160	170	180	190	200	210	220
Amphibious Ringer sol.	22	10	0	0	0	18	27	32	26	41	25	28	29	31	20	—	—	—	—	—	—	—
	34	9	6	0	0	0	29	27	36	48	40	41	36	32	34	38	36	32	—	—	—	—
Oxygenated amphibious Ringer sol.	23	14	0	0	3	0	6	0	0	2	3	0	0	0	0	0	0	0	—	—	—	—
	18	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	—	—	—	—
Oxygenated amphibious Ringer sol.—After 40' Ringer-glucose sol.	29	0	0	0	* 146	202	206	200	200	197	203	203	194	194	194	200	200	201	203	204	206	200
	16	14	0	0	119	200	200	206	210	212	212	212	202	204	198	196	196	198	200	200	194	197

* After 40 min. the perfusate was changed to oxygenated amphibious Ringer-glucose sol.
Each figure represents the glucose concentration (mg %) of the perfusate collected from the liver during periods of 10 minutes.

TABLE II. Effect of Alloxan upon Glycogenolysis of Isolated Liver of the Bull Frog Perfused with Ringer Solution without Oxygen.

Time in min.																	Wet wt of liver (g)	
10	20	30	40	50	60	70	80	90	100*	110	120	130	140	150	160	170	180	Before	After
27	6	0	0	0	18	20	21	23	31	21	20	12	10	12	6	0	—	15.0	15.3
11	0	0	2	0	12	24	27	26	9	13	7	10	0	0	0	0	—	19	19
31	16	6	0	0	0	19	28	38	18	9	0	0	0	0	0	0	—	18.6	19

* Indicates time when 10 mg alloxan were inj. into perfusate.

Each figure represents the glucose concentration (mg %) of the perfusate collected from the liver during periods of 10 minutes.

An initial period of perfusion with amphibious Ringer solution served to wash out any free blood and blood glucose. After 20′ to 30′ the fluid usually returned water-clear and sugar-free; then the specific experiment was started. The perfusate was collected at 10′ or 20′ intervals. The glucose determinations were done with the Nelson modification of the Folin-Wu Micro method(18).

Results. A) In a first series of experiments the spontaneous glycogenolysis of the isolated and perfused liver was studied, using as perfusates a) non-oxygenated, b) oxygenated amphibious Ringer solution and c) oxygenated amphibious Ringer-Glucose solution.

Table I represents the results of 2 experiments each. It can be seen that during an initial period of not more than 30′ decreasing amounts of glucose are obtained. During this time the perfusate is blood tinged and the glucose undoubtedly originates from the blood which is washed out. Afterwards the perfusate returns sugar-free for various periods of time. If non-oxygenated perfusion fluid is used (Exp. 1 and 2) glucose reappears in the perfusate about 30′ later and reaches a relatively stable level which is maintained for 2 to 3 hours. With the vascular tree now free of blood, this glucose must originate from the liver parenchyma and is evidence of spontaneous glycogenolysis. If O_2 is added to the perfusate the collected fluid remains practically free of glucose for the duration of the experiment, *i.e.*, 3 hours, indicating that with oxygen supply spontaneous glycogenolysis does not occur (Exp. 3 and 4). A glucose-Ringer solution saturated with O_2 returns from the liver with essentially unaltered sugar content (200 mg %) indicating that under these conditions neither glycogenolysis nor glycogenesis occurs unless both processes proceed at identical rates so as not to be noticeable from determinations of sugar-intake and output (Exp. 5 and 6).

B) The second group of experiments was undertaken to test the effect of a standard dose of alloxan (10 mg) on the spontaneous glycogenolysis of the surviving frog liver perfused with non-oxygenated amphibious Ringer solution. The liver was perfused for a period of 90 minutes before alloxan was injected. This initial period served not only to wash out any extraneous blood but also permitted the onset of spontaneous glycogenolysis.

Table II demonstrates that after the administration of alloxan the glucose content of the perfusate begins to decrease gradually until it finally disappears entirely. This inhibition of the spontaneous glycogenolysis is

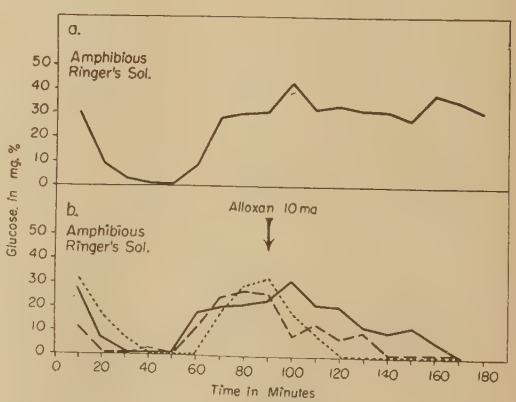

FIG. 1. a) Spontaneous glycogenolysis of isolated liver of bull frog perfused with amphibious Ringer solution without oxygen. b) Effect of alloxan on spontaneous glycogenolysis of isolated liver of bull frog perfused with amphibious Ringer solution without oxygen.

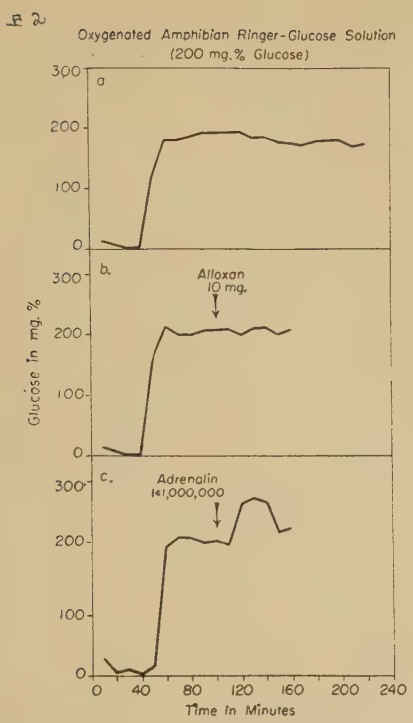

FIG. 2. Effect of adrenalin (1:1000000) and alloxan (10 mg) on isolated liver of bull frog perfused with oxygenated amphibious Ringer-glucose solution (200 mg %). During first 40 min. the liver is perfused with oxygenated Ringer solution without glucose.

noticed as early as 10′ after alloxan administration and lasts for more than one hour. Its reversibility cannot be deduced from the experiments presented since these were terminated while the inhibition persisted. Fig. 1 demonstrates graphically the spontaneous glycogenolysis of the perfused liver (a) and its inhibition by alloxan (b).

If alloxan is added to an oxygenated perfusate which, as shown, delays spontaneous glycogenolysis no evidence of its effect can be obtained. This is demonstrated in the following experiment (Fig. 2) where an oxygenated glucose containing perfusate was used. Fig. 2 shows that the amount of glucose recovered from the returning perfusion fluid was the same without (a) and with (b) alloxan administration. Fig. 2c shows in comparison the increase of the glucose return after adrenalin injection.

C) We now proceeded to investigate the effect of alloxan on the adrenalin-induced glycogenolysis. The following experiments were carried out: a) adrenalin 1:1,000,000 was injected into the abdominal vein 60′ after perfusion with oxygenated amphibious Ringer solution had been started. This resulted in the appearance of a significant amount of glucose in the returning perfusate for a period of 40′ to 60′. Afterwards the perfusate became again sugar-free. b) The administration of the same dose of adrenalin was followed after 20′ by an injection of 10 mg alloxan (1 cc of 1% solution in Ringer). The adrenalin glycogenolysis was not affected by this subsequent administration of alloxan. c) The same doses of adrenalin and alloxan were injected simultaneously. Glucose failed to appear in the returning perfusate. d) Alloxan administration preceded the injection of adrenalin. This experiment was car-

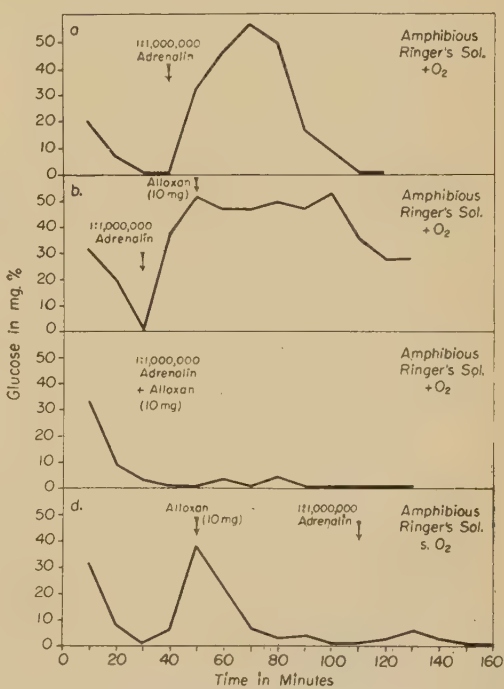

FIG. 3. a-c) Effect of adrenalin (1:1000000) and alloxan (10 mg) upon glycogenolysis of isolated liver of bull frog perfused with oxygenated Ringer solution.

a) Adrenalin-glycogenolysis. b) Alloxan administered 20′ after adrenalin does not affect adrenalin glycogenolysis. c) Alloxan administered simultaneously with adrenalin prevents adrenalin glycogenolysis. d) Alloxan inhibits spontaneous glycogenolysis and prevents adrenalin glycogenolysis in isolated liver of bull frog perfused with amphibian Ringer solution without oxygen.

ried out with non-oxygenated Ringer solution in order to demonstrate the alloxan inhibition of the spontaneous glycogenolysis. Adrenalin failed to reinduce glycogenolysis. Representative curves of these 4 types of experiments are shown in Fig. 3.

Discussion. Our finding that alloxan inhibits the spontaneous glycogenolysis as well as the adrenalin induced glycogenolysis of the perfused frog liver is in full agreement with the observations of Houssay and Gershman (9). The failure of Brunfeldt and Iversen (13) to obtain similar results rests probably in their technic. They apparently perfused under too great pressure and observed "rapidly occurring changes in the appearance of the liver and violent edematization." The difference between our findings and those of Kepinov(10) is a quantitative one only. In his experiments the inhibition of the spontaneous glycogenolysis was only partial and far shorter lasting than in ours. It is most likely that this can be explained as a species difference since he worked with the liver of a warm blooded animal.

The demonstration of an extrapancreatic effect of alloxan on carbohydrate metabolism is undoubtedly of significance for its diabetogenic action and particularly for the mechanism of the initial fluctuation of the blood sugar. It tends to weaken the evidence for the pancreatic origin of alloxan hypoglycemia. Whether the inhibition of hepatic glycogenolysis is its sole or a contributing cause will have to wait for further investigation. From the presented evidence the alloxan effect upon the liver appears to be an immediate one; the secondary hypoglycemic phase, on the other hand, becomes manifest not before 3 or 4 hours after alloxan administration. This time interval may be required until changes in liver metabolism reflect themselves in the periphery; yet, during this same period one usually observes a transitory hyperglycemic phase which has been considered by some to be due to adrenal stimulation(3,19). The finding that alloxan inhibits adrenalin glycogenolysis, however, tends to eliminate this mechanism as its cause.

Thus the acceptance of an hepatic origin of the alloxan hypoglycemia, far from solving the problem, opens again the question of the origin of the initial hyperglycemia.

Summary. 1. The technic for perfusion of the liver of the bull frog is described. 2. Alloxan inhibits the spontaneous glycogenolysis of the perfused liver. 3. Adrenalin glycogenolysis is inhibited by alloxan if given previously or simultaneously. 4. The significance of these findings for the alloxan-induced blood sugar fluctuations is discussed.

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The Electrocardiogram of the Normal Dog.* (20561)

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In the course of investigating cardiovascular changes produced by ventricular puncture in unanesthetized laboratory dogs, it became desirable to follow the electrocardiographic patterns over a period of time. For this purpose it was necessary to have a normal range of values against which to compare the variations found in order to determine whether they were significant. It was also essential to know whether the electrocardiograms from a single dog showed changes over a period of time under normal conditions, and if changes occurred the number of readings required on a dog to determine normality would have to be known. Search of the literature revealed no satisfactory criteria.

Smith(1) studied 66 dogs prior to ligation of the coronary arteries. The dogs were anesthetized and in an unstated position. Only standard limb leads were taken. No attempt at detailed analysis was made but it was stated the tracings were "fairly constant in conformation" and in the majority of cases the R wave in lead I was small, the T wave was small in all leads and not infrequently negative. Barnes and Mann(2) studied standard leads of normal unanesthetized dogs in an unstated position prior to coronary artery ligation and reported an extreme variation in T waves. No actual figures were made available. Katz *et al.*(3) studied serial electrocardiograms of standard leads on 3 normal dogs: trained, unanesthetized and on their right side. No data were presented but it was stated the tracings showed irregular fluctuations in form involving changes in amplitude and direction of all complexes, especially the T wave. These variations were neither progressive nor related to environmental factors and were considered normal, due to changes in the position of the heart. Harris and Hussey(4) studied electrocardiograms on 50 dogs in a control study prior to coronary artery ligation. The dogs were anesthetized with intraperitoneal amytal and were in an unstated position. No figures were presented but the authors report an "extreme variability of the so-called normal tracing." Lalick *et al.*(5) reported on 218 standard limb lead tracings on 24 normal trained unanesthetized dogs taken on either right or left side. Five dogs were examined on both sides. No detailed analysis of the tracings was made but it was reported that P waves showed changes in amplitude and direction in the same lead, that T waves were variable in amplitude and direction, and that changes in amplitude or disappearance of Q and S waves occurred. Even though variations occurred in various complexes in different animals the authors believed each dog's records were characteristic for that animal. Finally Petersen and colleagues(6) set up detailed criteria for standard and augmented unipolar limb leads for normal young beagle dogs untrained and unanesthetized in the supine position. Tracings were repeated in the same animal once in 4 instances, twice in 5, and 4 times in one instance. From the repeated tracings it was concluded that the variations in serial electrocardiograms were more marked than in man but not so great as to preclude comparison with one another. Although Petersen's study presented a table of normal values for comparison, the data were accumulated on a single breed and of smaller size than used in our laboratory. No standard deviation was calculated nor was the RS deflection measured nor the P wave except in lead II. The number of tracings on a single dog was insufficient for statistical analysis.

This report concerns the detailed analysis of 62 electrocardiograms on 30 normal laboratory dogs of mongrel breed and indeterminate age. Standard limb leads and augmented unipolar extremity leads are reported. The object of the analysis is to establish a

* The authors are indebted to Miss Shirley J. Hodgins for help in the collection of material, and to Mrs. Frances B. Locke for her invaluable assistance in the statistical analysis of the data.

TABLE I. Electrocardiographic Standards.*

		Leads†					
		I	II	III	AVR	AVL	AVF
P	Mean	.17	.27	.16	−.20	.02	.19
	S.D.	.07	.09	.11	.09	.10	.11
	—Max	.40	.50	.39	.19	.24	.45
	Min	.05	.03	−.27	−.35	−.16	−.10
	% incidence	100	100	100	100	90	100
Q	Mean	.16	.17	.13	.20	.11	.17
	S.D.	.24	.17	.12	.45	.18	.14
	—Max	1.15	.89	.44	1.89	1.27	.70
	Min	0	0	0	0	0	0
	% incidence	70	97	77	20	43	78
R	Mean	1.05	1.77	1.11	.21	.41	1.35
	S.D.	.58	.48	.48	.24	.40	.50
	—Max	2.22	3.09	2.43	1.28	1.79	2.52
	Min	.20	.30	.14	.02	.02	.10
	% incidence	100	100	100	100	97	100
R′	Mean				.06		
	S.D.				.11		
	—Max	.03	.19	.24	.39	.38	.23
	Min	0	0	0	0	0	0
	% incidence	3	13	10	40	23	13
S	Mean	.09	.09	.23	1.21	.30	.17
	S.D.	.14	.18	.31	1.27	.10	.17
	—Max	.80	.79	1.48	2.34	2.58	.67
	Min	0	0	0	0	0	0
	% incidence	47	60	70	89	67	70
R-S	Mean	1.14	1.92	1.39	1.46	.80	1.54
	S.D.	.56	.59	.45	.68	.66	.51
	—Max	2.62	3.09	2.58	2.85	3.41	2.97
	Min	.27	.30	.33	.20	0	.24
	% incidence	100	100	100	100	97	100
T	Mean	.05	.18	.13	−.11	−.04	.13
	S.D.	.17	.26	.23	.20	.14	.23
	—Max	.63	.74	.81	.26	.36	.74
	Min	−.36	−.27	−.26	−.60	−.40	−.25
	% incidence	100	100	100	100	100	100

	P duration	P-R interval	QRS duration	Q-T interval	Wt	Heart rate
Mean	.07	.12	.06	.21	17.9	121
S.D.	.02	.02	.01	.02	3.7	19
Max	.12	.15	.08	.28	32.7	162
Min	.04	.10	.04	.16	12.8	72

* Data from 30 dogs.
† Amplitudes expressed in millivolts and duration in sec.

normal range of values for the average laboratory dog and to set the number of tracings required on a dog to determine normality.

Method. The animals were trained and unanesthetized. All electrocardiograms were taken in the supine position. A direct writing machine was used. On 3 dogs 10 tracings were taken at different times over a period of 5 months, on one dog 4 tracings, on 2 dogs 2 tracings.

Results. The data for the 30 dogs are presented in Table I. In those instances where more than one tracing per dog was done, an average was taken of all figures for that animal. In Table II are presented the data on one of the 3 dogs on whom 10 tracings were taken. Table III indicates the direction of T wave deflections and the electrocardiographic position of the heart as defined by Wilson *et al.*(7) for the group dogs and on each of the 3 on whom 10 tracings were taken. All animals showed a sinus arrhythmia except

TABLE II. Serial Electrocardiographic Readings.*

		Leads†					
		I	II	III	AVR	AVL	AVF
P	Mean	.19	.36	.21	−.23	.05	.28
	S.D.	.03	.08	.09	.03	.03	.07
	—Max	.24	.50	.39	−.19	.07	.45
	Min	.15	.21	.10	−.28	0	.19
	% incidence	100	100	100	100	90	100
Q	Mean	.32	.15			.54	.04
	S.D.	.38	.15			.40	.07
	—Max	1.15	.51			1.27	.19
	Min	0	0			0	0
	% incidence	90	80	10	10	90	30
R	Mean	.94	1.92	1.36	.25	.66	1.61
	S.D.	.28	.12	.29	.26	.51	.14
	—Max	1.43	2.10	1.75	.89	.90	1.78
	Min	.63	1.73	.85	.07	.44	1.39
	% incidence	100	100	100	100	100	100
R′	Mean						
	S.D.						
	—Max				.15		
	Min				.04		
	% incidence	0	0	0	.50	0	0
S	Mean		.30	.74	1.19		.51
	S.D.		.12	.17	.46		.12
	—Max		.48	.93	1.63		.67
	Min		.13	.36	0		.35
	% incidence	0	100	100	90	0	100
R-S	Mean	.94	2.22	2.09	1.51	.66	2.21
	S.D.	.28	.12	.36	.59	.51	.34
	—Max	1.43	2.35	2.58	2.58	.90	2.97
	Min	.63	2.00	1.31	.22	.44	1.73
	% incidence	100	100	100	100	100	100
T	Mean	−.10	.29	.42	−.08	−.22	.33
	S.D.	.17	.27	.20	.21	.14	.22
	—Max	.23	.60	.81	.26	.05	.74
	Min	−.36	−.25	.19	−.28	−.40	.26
	% incidence	100	100	100	100	100	100

	P duration	P-R interval	QRS duration	Q-T interval	Wt	Heart rate
Mean	.06	.13	.05	.19	18.1	120
S.D.	.01	.01	.01	.01		17
Max	.08	.14	.06	.20		151
Min	.04	.11	.04	.18		104

* Ten consecutive readings on one dog.
† Amplitudes expressed in millivolts and duration in sec.

for one who demonstrated normal sinus rhythm. One of the dogs on whom 10 tracings were taken had occasional ventricular premature contractions on one electrocardiogram and occasional auricular premature contractions on another. The S-T segment was isoelectric in every instance.

Smith(1) noted that when the T wave was inverted in all leads (standard limb) the mortality "seemed to be high" within 24 hours after operation for ligation of the coronary arteries. In our series 3 dogs had inverted T waves in all standard leads. One of these 3 had an upright T in ${}_{A}V_{R}$ and inverted in ${}_{A}V_{L}$ and ${}_{A}V_{F}$; one had upright T in ${}_{A}V_{R}$ and ${}_{A}V_{L}$ and inverted in ${}_{A}V_{F}$; the third had diphasic T in ${}_{A}V_{R}$ upright in ${}_{A}V_{L}$ and inverted in ${}_{A}V_{F}$.

Lalick(5) observed that in 78% of records which demonstrated a Q wave in lead I an inverted T_I was present. In our series there were 22 tracings which showed a Q_I of 1 mm

TABLE III. Direction of T-Wave Deflections (in %).

		I	II	III	AVR	AVL	AVF	Heart positions,* %
Group control dogs	Upright	60	70	70	23	33	70	S.H. 33
	Inverted	3	27	30	66	63	23	I. 3
	Diphasic	37	3	0	10	3	7	S.V. 63
Ginger	Upright	10	100	100	10	10	80	S.H. 90
	Inverted	70	0	0	70	90	0	I. 10
	Diphasic	20	0	0	20	0	20	S.V. 0
Lucky	Upright	100	90	40	0	80	60	S.H. 10
	Inverted	0	0	0	90	0	0	I. 10
	Diphasic	0	10	60	10	20	40	S.V. 80
Sandy	Upright	60	80	100	0	10	90	S.H. 90
	Inverted	30	0	0	80	0	0	I. 10
	Diphasic	10	20	0	20	90	10	S.V. 0

* S.H. = semi-horizontal; I. = intermediate; S.V. = semi-vertical.

or larger. In 18 of these or 82% an inverted T_I was present also. Lalick also reported that when Q_I was greater than 4 mm, over 90% was followed by an inverted T. In our 62 tracings 8 demonstrated a Q_I greater than 4 mm and each was followed by an inverted T except one, which showed a diphasic T with the inverted component first 1.8 mm deep and the upright component 0.5 mm high. In Lalick's tracings where an S wave was present in lead III it was followed by an upright T 75% of the time. In our series there were 37 instances of an S_{III} of 1 mm or greater, 31 of these or 84% were followed by an upright T.

Comment. The best procedure to obtain a normal range is to take several readings on many dogs. In our series only single readings were taken on most dogs. Additional readings would have made the frequency distribution more smooth, but probably would not have altered the normal range limits to any extent. From a statistical standpoint the data obtained are satisfactory to establish a normal range. Although the values are not normally distributed the normal range seems to be adequately described for P, R, and T readings by taking the mean plus and minus 2.5 standard deviations; this will leave 1% of the normal values which will fall out of this range. Depending upon the desired conservatism other numbers of standard deviations might be used. For Q and S magnitude where zero values are frequent the range is better described by taking zero plus a number of standard deviations. For example, to establish a range including 99% of the normal values, instead of taking the mean plus and minus 2.5 standard deviations, the range would be 0 plus 5.4 standard deviations. Variations in tracings on the same dog taken at different times are as great as among different dogs. The results of analysis of the variance test applied to 10 successive readings on each of 3 dogs for the value of P_I reveals no significant difference between the variation found in the same dog and that found among the 3 dogs. Because of the variation found within the same animal it is preferable to use the mean of 3 tracings in order to reduce the effect of the occasional extreme value.

Summary. 1. A table of values is presented for the electrocardiogram of the average normal laboratory dog; trained, unanesthetized, and in the supine position, based on data from 62 tracings on 30 dogs. 2. Data obtained from 10 serial tracings on each of 3 dogs indicates a variation within the same animal not significantly different from that obtaining among different animals. 3. It is recommended that to establish the normal electrocardiogram of a dog a mean of 3 tracings taken at different times be used to reduce the effect of the occasional extreme value.

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Peripheral Blood Concentrations of Steroids in Man After Oral Administration of 17-Hydroxycorticosterone.* (20562)

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Recent developments in chromatographic and micro-analytical technics for the resolution and quantitation of steroids make possible the analysis of corticosteroids in peripheral blood of man. A method for analyzing 17-hydrocorticosteroids in peripheral blood has been developed by Nelson and Samuels(1). They have employed Florosil for resolution and a modified phenylhydrazine reaction(2) for quantitation of the steroids. The method of Sweat(3) utilizes silica gel for resolution and sulfuric acid fluorescence for quantitation of corticosterone-like steroids and 17-hydroxycorticosterone. In the present study the technic of Sweat has been applied to the determination of the concentrations of steroids in peripheral blood of man following the oral administration of a single dose of 17-hydroxycorticosterone.

Methods. Eight normal adult males were subjects for this study. Immediately following withdrawal of a control blood sample, 6 of the subjects received 50 mg of 17-hydroxycorticosterone (Hydrocortisone, Merck) orally; blood samples were collected at various time intervals thereafter for steroid analysis. In control subjects, blood samples were collected and analyzed, but no hormone was given. Twenty ml of blood were drawn from an antecubital vein, mixed with ⅓ ml of heparin and centrifuged. Ten ml of separated plasma were analyzed for steroid content. The method employed for analysis of corticosterone-like steroids and 17-hydroxycorticosterone in plasma was that developed by Sweat. In brief, this method consists of a) extraction of the plasma sample with 10 volumes of chloroform, b) fractionation of the dried chloroform extract between 70% ethanol and petroleum ether and c) resolution on a silica-gel column by elution with mixtures of ethanol and chloroform in various ratios. The resolved steroids were quantitated by the degree of fluorescence developed in concentrated sulfuric acid. Recovery studies have shown that approximately 95% of 17-hydroxycorticosterone and 80% of corticosterone added to plasma are resolved and recovered by this technic.

Results. The individual values obtained for the concentration of 17-hydroxycorticosterone are plotted in Fig. 1. Curve A is a line of best fit based on results obtained in 5 individuals who received 50 mg of 17-hydroxycorticosterone orally. A significant increase in plasma concentration of the hormone occurred 15 minutes following its administration. The peak concentration was attained between 45 and 60 minutes; normal values were reached between 4 and 6 hours. Curve

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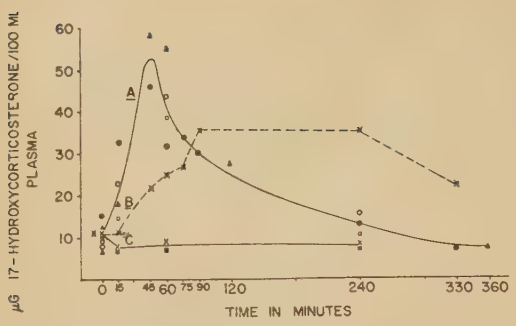

FIG. 1. Plasma concentrations of 17-hydroxycorticosterone in peripheral blood of humans. Curves A and B represent subjects who received 50 mg of 17-hydroxycorticosterone orally. Curve C represents control subjects who received no hormone.

B represents the concentrations of 17-hydroxycorticosterone in a subject who received 50 mg of 17-hydroxycorticosterone orally and whose pattern of response appeared to be quite distinct from that of the other 5 subjects. Results obtained from the two control subjects are represented by curve C. During the 4 hours of collection there was no significant change in plasma concentration of 17-hydroxycorticosterone in these 2 individuals.

Analyses for the corticosterone-like steroids present in human plasma were run simultaneously with the 17-hydroxycorticosterone determinations. In the 6 test subjects the control values for the corticosterone-like steroids were between 2.5 and 5.9 μg per 100 ml of plasma. Following the oral administration of 17-hydroxycorticosterone to these individuals, no significant increase in the plasma concentration of the corticosterone-like steroids occurred. No significant change occurred in the concentration of this fraction in the control subjects during a 4-hour collection period.

Discussion. The method of Sweat for blood steroid analysis effectively resolves corticosterone-like steroids and 17-hydroxycorticosterone on a silica-gel column. A number of evidences have been accumulated(4) which strongly suggest that the more polar fraction which fluoresces is 17-hydroxycorticosterone. Other evidences suggest that the material which chromatographs in the corticosterone region and which fluoresces consists of corticosterone and possibly other steroids. Nelson *et al.*(5) have reported blood levels of 17-hydroxycorticosteroids in humans following the oral administration of 50 mg of 17-hydroxycorticosterone acetate. In general, the time pattern of increase and decrease of blood steroid concentrations obtained by these authors appears to be in essential agreement with the data presented in this paper.

In 5 of 6 individuals given 17-hydroxycorticosterone in the present study, there was a rapid increase in plasma concentration of this steroid with maximal levels being attained 45 to 60 minutes following its oral administration. No significant elevation in plasma steroid concentration could be detected after 4 hours. When 17-hydroxycorticosterone is given orally frequent administration is necessary for maintenance of elevated plasma concentrations. In a single individual given oral 17-hydroxycorticosterone (curve B, Fig. 1), a slower increase in plasma concentration of this steroid was observed with the maximal level occurring between 1.5 and 4 hours. These results can best be explained by delayed absorption of the steroid. If the major absorption pathway is via the hepatic portal venous system, results obtained in this study would imply that an appreciable fraction of orally administered 17-hydroxycorticosterone passes through the liver without structural alteration. The possibility exists that 17-hydroxycorticosterone passes from the gastro-intestinal tract into the blood stream by way of the lymphatic system. However, Dull(6), in preliminary studies, has been unable to detect cortisone in thoracic duct lymph subsequent to the introduction of the steroid into the intestinal tract of the dog.

Summary. A method which measures blood concentrations of corticosterone-like steroids and 17-hydroxycorticosterone has been employed to determine the plasma concentrations of these steroids in humans following a single oral dose of 17-hydroxycorticosterone. A rapid increase in plasma concentration of this steroid occurred in 5 of 6 subjects; maximal levels were attained between 45 and 60 minutes after administration. The plasma concentration of this steroid decreased rapidly thereafter, reaching normal values between 4 and 6 hours. No significant elevation in plasma concentration of the corticosterone-like steroids could be detected following the

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Effect of Thyroidectomy on Adrenal Weight in Adult Male Rats. (20563)

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There has been some discrepancy in the literature concerning the effect of thyroid deficiency (affected by thyroidectomy or the administration of goitrogens) on adrenal weight. Leblond and Hoff(1) noted a decrease in the size of the adrenals in rats receiving goitrogenic sulfonamide drugs or thiouracil. This was confirmed by Baumann and Marine(2) when they noted involution of the adrenals to half their former size in rats fed thiouracil for 4 months. Zarrow and Money(3) have shown that after 6 weeks or more of thyroid deficiency there is a relative as well as an absolute loss of adrenal weight. Freedman and Gordon(4) reported that thiouracil treatment and thyroidectomy in rats results in adrenal atrophy. On the other hand, Winter and Emery(5) reported that, in rats, thyroidectomy *per se* does not appreciably influence the size of the adrenals, nor does it affect the degree of compensatory adrenal hypertrophy as compared with intact animals. Morris(6) reported that after treatment with thiouracil or thyroidectomy the cockerel shows a marked adrenal hypertrophy, particularly when adrenal weights are calculated on a relative weight basis. The adrenals of young, thyroidectomized rats were found to be similar in weight to those of intact animals when calculated on a relative basis(7). The administration of the goitrogen p-aminosalicylic acid to rats was without effect on adrenal weight(8). The use of Amphenone "B", which inhibits the incorporation of radioiodine into thyroid protein as effectively as propylthiouracil, produced an adrenal enlargement in rats(9).

Since thyroid deficiency in the young growing animal results in the retardation of body growth, the adrenal atrophy of hypothyroidism cited by some investigators may be a reflection of the reduced body weight. This study was designed with that problem in mind. To rule out the effect of thyroid deficiency on growth, adult, fully grown ("plateaued") rats were utilized.

Methods. Male rats of the Holtzman strain were used throughout this study. Animals were made thyroid deficient by total thyroid-parathyroidectomy at an age of 5½ months, and together with controls, permitted to take Purina Laboratory Chow pellets and tap water *ad libitum* for an additional 2 months. At autopsy, organs were weighed on a torsion balance and the pituitary and adrenal glands saved for histological study.

Results. Although all of the animals had reached a "plateau" in their growth curves, there is still a small, but significant, difference in the body weights of thyroidectomized rats compared with their controls. The thyroidectomized animals weighed about 34 g less than the unoperated ones (Table I). Even with this difference in body weight, the absolute weight of the right adrenals of the thyroidectomized rats did not differ significantly from that of the intact animals. Neither left nor right adrenal glands showed any difference in relative weights between the 2 groups (Table I). There is, however, a significant

TABLE I. Effect of Thyroidectomy on Adrenal, Thymus, and Preputial Gland Weight of Adult Male Rats.

Group	No. of rats	Body wt (g)	Left adr. wt (mg) Abs.	Left adr. wt (mg) Rel.*	Right adr. wt (mg) Abs.	Right adr. wt (mg) Rel.	Thymus wt (mg) Abs.	Thymus wt (mg) Rel.	Preputial wt (mg) Abs.	Preputial wt (mg) Rel.
Control	8	486 ± 7†	26.2 ± .9	5.4 ± .2	24.0 ± 1.3	5.0 ± .3	315 ± 14	64.7 ± 2.6	183 ± 24	37.8 ± 5.1
Thyroidectomized 2 mo	17	452‡ ± 6	22.0‡ ± .6	4.9 ± .1	20.4 ‡ .5	4.5 ± .1	283 ± 21	62.3 ± 4.1	159 ± 21	35.3 ± 4.8

* mg/100 g body wt. † Stand. error. ‡ Difference from controls statistically significant.

TABLE II. Effect of Thyroidectomy on Gonadal and Secondary Sex Organ Weight of Adult Male Rats.

Group	No. of rats	Body wt (g)	Left testis wt (mg) Abs.	Left testis wt (mg) Rel.*	Seminal vesicles wt (mg) Abs.	Seminal vesicles wt (mg) Rel.	Ventral prostate wt (mg) Abs.	Ventral prostate wt (mg) Rel.
Control	8	486 ± 7†	1835 ± 20	378 ± 7	548 ± 25	113 ± 6	1033 ± 85	213 ± 18
Thyroidectomized 2 mo	17	452‡ ± 6	1916 ± 40	424‡ ± 10	679‡ ± 32	151‡ ± 8	1054 ± 39	234 ± 10

* mg/100 g body wt. † Stand. error. ‡ Difference from controls statistically significant.

difference in the absolute weights of the left adrenal glands in the operated and unoperated animals.

In conformity with a previous publication (10), the left adrenals of the animals in this study also were heavier by about 8% than the right ones.

There was no difference in the weights of the thymus and preputial glands of the thyroidectomized and intact rats (Table I).

The relative weights of the left testes and seminal vesicles of the thyroidectomized animals were greater than those of intact rats. The seminal vesicles of operated animals were heavier than controls even on an absolute weight basis (Table II). Although the weights of the ventral prostate of thyroidectomized rats were larger, this difference was not significant.

Discussion. The difference in body weight between the thyroidectomized and intact adult animals, though significant, was small and is far less striking than a similar comparison made on young growing animals. The young intact animal weighs nearly twice as much as its thyroidectomized counterpart(7). The effect of the absence of thyroid hormone in rats which have reached adulthood (a "plateau" in the growth curve) is not nearly as noticeable in relation to body weight.

Examination of the pituitary glands of thyroidectomized and intact rats insured completeness of the operation in the former group. The operated animals exhibited a complete degranulation of pituitary acidophile cells and an increase in number and size of the basophiles following the staining technic of Briseno and Finerty(11).

The data on adrenal weight support the viewpoint that thyroid deficiency does not cause adrenal atrophy. Certainly there is no indication that the adrenal gland of a thyroidectomized rat is subnormal in function. It has been reported that the adrenal of a thyroid-deficient rat responds to stress, as indicated by adrenal ascorbic acid, with depletions greater than under normal conditions (7). Freedman and Gordon(12) reported no impairment of the pituitary-adrenal mechanism of thiouracil-treated rats exposed to cold. The adrenals of thiouracil-treated or thyroid-

ectomized rats are just as responsive to ACTH as a normal gland(7,13).

There is no reason to assume atrophy of the adrenal of a thyroid deficient animal on the basis of a decreased ACTH secretion from the pituitary gland. Both Halmi and Bogdanove(14) and Hess and Finerty(7) found no significant difference in ACTH content of intact and thyroidectomized rat pituitary glands.

If thyroid deficiency causes adrenal atrophy, there should be some indication of adrenal hypofunction. However, such is not the case. It has been reported that adrenal deficient rats have larger preputial glands(15). On the basis of this study as well as that of a previous report(15), the preputials of thyroidectomized rats are equivalent in weight to those of intact animals. Adrenal deficient rats have larger thymus glands than normal animals(16,17). There was no significant difference between the weights of the thymus glands of thyroidectomized and unoperated animals. Therefore, there is no reason for assuming adrenal atrophy as a consequence of thyroid deficiency.

The adrenal of a thyroidectomized rat, however, does have a histological appearance which differs from that of intact animals. When frozen sections are stained with Sudan IV, the adrenal of a thyroidectomized rat exhibits a somewhat narrower zona fasciculata than in controls, with the fat restricted almost entirely to the outer part of this zone. The fat stains a much deeper red in these glands. This observation has also been made previously by Deane and Greep(18) and Hess and Finerty(7).

The enlargement of the testes and seminal vesicles of the thyroidectomized rats was not surprising. The increased sensitivity of the gonads and secondary sex organs of the thyroidectomized animal to gonadotrophins has been reported previously. Schockaert(19) has shown that the enlargement of the secondary sex organs in male rats given anterior pituitary injections is significantly increased by thyroidectomy. The greatest gonadal response secured by Smith and Engle(20) from pituitary implants was in a thyroidectomized animal. Fluhmann(21) reported that thyroidectomy was followed by greater than normal ovarian responses to anterior pituitary extracts, which was confirmed by Tyndale and Levin(22) in their experiments utilizing injected gonadotrophic material.

Summary. 1. Although thyroidectomized adult male rats, having reached a "plateau" in their growth curves, exhibit a small difference in body weight when compared with controls, there is no significant difference in the relative weights of the adrenal glands between the 2 groups. The absolute weights of the right adrenals of the operated and intact animals showed no difference. It is concluded, therefore, that thyroidectomy does not cause adrenal atrophy. Evidence for the normal function of adrenal glands of thyroid deficient rats is discussed. 2. The enlarged testes and seminal vesicles of the thyroidectomized rats are in conformity with previous observations that there is an increased sensitivity of the gonads and secondary sex organs of thyroid deficient rats to gonadotrophic hormones.

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Arterial Pressures in Street Dogs: Incidence and Significance of Hypertension. (20564)

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Spontaneous hypertension in dogs might more closely resemble the "essential" hypertension of human beings than experimental neurogenic or nephrogenic hypertension. The condition has been described (Stamler, Katz, Rodbard(1)), but authorities(2) disagree as to its incidence and significance and agree only that it occurs infrequently. The present report is based on arterial pressure measurements in 400 dogs selected at random, most of them adult mongrels.

Methods. Pressures were measured in a sound-proofed room while the dogs were gently restrained on their backs. The femoral artery was punctured with a 20 g needle connected to a mercury manometer by rigid tubing filled with 5% citrate solution. Except as noted below, only one measurement was made and this usually within a few days after the dogs had been received; thus the data do not reflect the effect of training. The one observer was skilled, so that few dogs resisted the procedure and most lay quietly after a few minutes of petting. Measurements were continued until the pressure reached what seemed a minimum level; this usually required only 4 or 5 minutes but was sometimes prolonged up to 15 or 20 minutes.

Results are summarized in Table I. Arterial pressures of most of the animals (319/400) were in the range 111-140 mm Hg. Some ranged from 141 to 150 mm Hg and a few between 99 and 111 mm Hg. Only 9 (2% of the group) could be considered hypertensive, *i.e.*, if one accepts the arbitrary definition of an arterial pressure higher than 150 mm Hg. The arterial pressures of 7 of these 9 ranged between 150 and 157 mm Hg at the first determination and fell below this level when measurements were repeated on succeeding days and "training" established. Only 2 of the 400 showed appreciable hypertension with arterial pressures of 175 and 205 mm Hg which persisted at these respective levels with repeated testing over several weeks.

Since these two might be considered examples of spontaneous hypertension, they were studied more closely. One, dog A, was old; at postmortem, its kidneys were contracted and had the gross appearance of chronic pyelonephritis; the renal pelves of dog B were found at postmortem to contain stones and sections of the kidney demonstrated nephrosclerosis on microscopic examination. Respective renal plasma clearances of p-aminohippurate were 8.3 and 8.7 cc per kg body weight per minute and the simultaneous plasma clearances of exogenous creatinine 2.7 and 2.8. These clearance rates are lower than the means of 13.5 (effective renal plasma flow) and 4.3 (glomerular filtration rate) found in normal dogs (Houck(3)).

Previous evidence for the occurrence of spontaneous hypertension is mainly the rela-

TABLE I. Range of Arterial Pressure in 400 Street Dogs.

Mean systolic pressure in mm Hg	100–110	111–120	121–130	131–140	141–150	151–156	175	205
No. of dogs	19	82	123	114	53	7	1	1

tively normal renal clearances of 3 dogs with sustained elevation of arterial pressure studied by Stamler, Katz and Rodbard(1); the clearance rates of one of these were 10.2 (effective renal plasma flow) and 3.5 (glomerular filtration rate) cc per kg per minute. No data are available as to the appearance of the kidneys of this animal; however, the kidneys of the other two, whose renal plasma clearances were somewhat more depressed below mean normal did show "slight to moderate chronic focal lesions". Thus, renal plasma clearances while within the range of normal have been found to lie below the normal mean in 5 dogs with seeming spontaneous hypertension; 4 of these for which data are available demonstrated renal lesions at autopsy.

Focal lesions, such as occur in some dogs with experimental renal hypertension, do not necessarily depress plasma clearances (Corcoran and Page(4)) so that normal rates of plasma clearance are observed in some dogs with experimental renal hypertension(1,4). Demonstration of normal or slightly subnormal rates of plasma clearance in dogs found to be hypertensive does not therefore establish that the hypertension is of extrarenal origin. Actually, focal or diffuse renal lesions are not uncommon in dogs (Bloom(5)), and it would be surprising if these did not sometimes elicit renal hypertension. Hamilton *et al.*(6) observed that dogs with hyalinized arterioles and with glomerular nephritis have higher pressures than the average population.

Summary. Hypertension resembling "clinical essential" hypertension may occur in dogs, but the weight of the evidence inclines us to the view that this condition must be very rare; what seems to be "spontaneous" canine hypertension is usually attributable to renal disease. The relatively narrow spread of arterial pressures in this series of street dogs indicates that the skill of the observer is at least as important as the training of the dog.

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Action of 3-Acetyl-Strophanthidin in Isolated Mammalian Heart.* (20565)

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An ester of strophanthidin, 3-acetyl-strophanthidin, has been found to be effective for slowing ventricular rate in auricular fibrillation and for treating paroxysmal tachycardia. After intravenous administration, the onset of action is rapid and the duration of effect is relatively short(1). However, the positive inotropic action of 3-acetyl-strophanthidin has been questioned by Wedd and Blair(2) who found marked differences in the response of the turtle heart to digitalis and to 3-acetyl-strophanthidin. From their results, the authors concluded that this ester cannot be an

* We are indebted to Messrs. Thomas Lenahan and Darrell Caldwell for their capable assistance in these experiments.

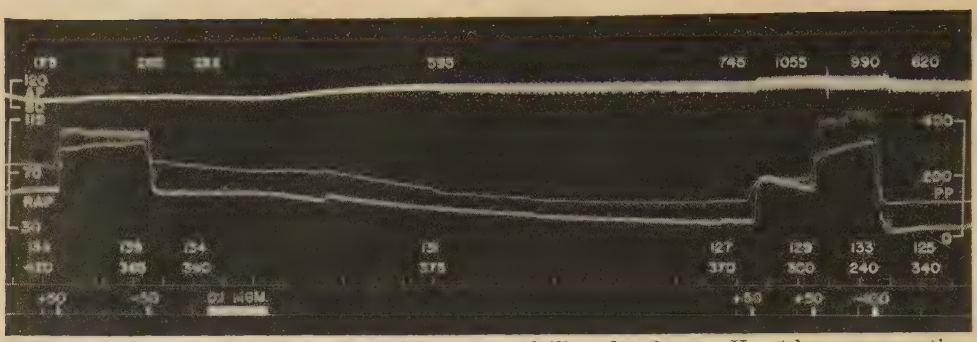

FIG. 1. Action of 3-acetyl-strophanthidin on the failing dog heart. Heart-lung preparation. Wt of heart-lung dog, 11.4 kg. Approximate blood vol 850 ml. Arterial resistance 48 mm mercury. Tracings from top to bottom: arterial blood pressure (scale on left in mm of mercury); pulmonary pressure (scale on right in mm of water); right auricular pressure (scale on left in mm water); time in 1-min. intervals; signal. Horizontal rows of figures from top to bottom: systemic output in ml/min., heart rate per min., reservoir vol in ml, and changes in inflow level in mm. At signal, 0.100 mg 3-acetyl-strophanthidin inj.

effective cardiac drug with digitalis-like action. Using the cat papillary muscle preparation, Greiner and Reilly(3) were able to demonstrate that 3-acetyl-strophanthidin has a positive inotropic action and that, mole for mole, it is more effective than strophanthidin. They concluded that the clinical use of 3-acetyl-strophanthidin is justified and that the effects seen in patients are due to its rapid digitalis-like action.

Although Greiner and Reilly employed mammalian heart muscle, they did not study the effect of the drug on the entire heart. Since the question of the presence or absence of a positive inotropic action of 3-acetyl-strophanthidin is of major importance for its clinical use, experiments were performed using the heart-lung preparation of the dog in order to compare the cardiac action of this ester with that of ouabain.

Method. Mongrel dogs of either sex, weighing from 8.9 to 12.4 kg, were used in all experiments. The animals were fasted for 18 hours and were then anesthetized with Secobarbital Sodium.† Heart-lung preparations were made as described by Krayer and Mendez(4). Systemic output was measured with a rotameter‡ and the float level read visually each minute. Heart rate was recorded on an ink-writing oscillograph. The initial total blood volume varied from 800 to 900 ml. Heart failure was produced by the administration of 50 to 80 mg of Secobarbital Sodium injected into the inflow tubing in divided doses. Ouabain and 3-acetyl-strophanthidin§ were prepared as 1:1000 stock solutions in 47.5% ethanol. Fresh dilutions of the stock solutions were made daily in 0.9% sodium chloride solution.

Results. Two experiments were performed with ouabain and four with 3-acetyl-strophanthidin. That 3-acetyl-strophanthidin does produce a positive inotropic effect in the failing dog heart can be seen clearly in Fig. 1. During the production of cardiac failure the systemic output had decreased from 840 to 285 ml/min and both the right auricular and pulmonary arterial pressures had increased greatly. Within 2 minutes after the injection of 100 μg of 3-acetyl-strophanthidin into the inflow tubing, the right auricular pressure began to decrease and the systemic output increased. Ten minutes after the injection the right auricular and pulmonary pressures had returned nearly to normal and the systemic output had increased to above its initial value. Although the ability of the heart to compensate for an increased work load (increased venous inflow) was not completely restored to its initial status, it was greatly improved.

In Table I are summarized 2 experiments in which repeated doses of ouabain or of 3-acetyl-strophanthidin were administered until toxic

† 'Seconal Sodium' (Secobarbital Sodium, Lilly).

‡ The rotameter was made by C. Wilson of the Lilly Clinic.

§ The 3-acetyl-strophanthidin was prepared in our laboratories by Mr. John S. Welles.

TABLE I. Effect of Repeated Doses of Ouabain or 3-Acetyl-Strophanthidin on the Isolated Dog Heart.

Time, min.	Systemic output, ml/min.	Heart rate/min.	Arterial pressure, mm Hg	Pulmonary pressure, mm H_2O	Right auricular pressure, mm H_2O	Competence index*	Reservoir vol, ml	Blood temp., °C
				Experiment 31				
5	595	137	95	65	35.0		630	38.2
8						.90		
15	Secobarbital sodium—50 mg							
35	"	"	20 "					
53	"	"	10 "					
70	"	"	10 "					
74	265	126	79	175	68.5		485	38.0
77						.24		
82	280	125	80	180	68.5		470	37.9
83	O†—50 μg							
92	285	123	83	160	63.5		470	38.0
93	O — 20 μg							
100	330	122	87	150	60.5		465	38.0
101	O — 50 μg							
110	415	121	93	125	53.5		460	38.1
111	O — 50 μg							
116	480	107	96	105	46.5		460	38.4
117	C†							
				Experiment 27				
3	675	131	109	60	29.5		520	38.0
12						.93		
26	Secobarbital sodium—50 mg							
39	"	"	50 "					
43	330	136	102	170	66.5		350	37.5
45						.09		
48	335	136	100	175	73.5		305	37.2
49	3-A†—50 μg							
63	470	132	108	100	55.5		350	37.9
65						.35		
72	510	131	110	90	50.5		360	38.0
73	3-A — 50 μg							
82	605	128	113	60	43.5		380	38.0
84						.89		
93	595	129	114	55	40.0		335	37.8
94	3-A — 50 μg							
97	595	128	113	50	39.0		320	38.0
99	C†							

* Competence index (5) is the ratio: $\frac{\text{Increase in inflow level—increase in right atrial pressure}}{\text{Increase in inflow level}}$

Although both 50 and 100 mm increases in inflow level were used in the experiments, only the effects caused by the 50 mm rise were used to compute the competence index.

† O = Ouabain; 3-A = 3-Acetyl-strophanthidin; C = Cardiac irregularities started.

effects (as evidenced by cardiac irregularities) were produced. A total dose of 170 μg of ouabain was necessary, under these circumstances, to produce cardiac irregularities. When 3-acetyl-strophanthidin was administered to another preparation in a similar manner, cardiac irregularities began after administration of 150 μg. Although only one experiment has been performed with each substance administered in this manner, it is clear that they are very similar with regard to potency, toxicity, and ratio between therapeutic and toxic doses.

The dose producing a clear-cut positive inotropic action is about 75 μg for ouabain and 100 μg for 3-acetyl-strophanthidin. In the second experiment with ouabain, cardiac irregularities, beginning about 20 minutes after administration of the glycoside, were produced by 75 μg.

When 3-acetyl-strophanthidin was used, irregularities were produced by 150 μg in each

of 3 experiments and 350 μg in the fourth.

Discussion. The cardiac action of 3-acetyl-strophanthidin has been shown to be a positive inotropic one in the dog heart-lung preparation. This confirms the positive inotropic response of the cat papillary muscle found by Greiner and Reilly(3), and indicates that the therapeutic action of the drug in man is similar to that of digitalis. Apparently, the cold-blooded turtle heart used in the experiments of Wedd and Blair(2) reacts in a different manner to different cardiac drugs than does the warm-blooded mammalian heart.

The experiments reported here are not sufficient in number to permit any quantitative comparison of the activity of ouabain and 3-acetyl-strophanthidin, but the results indicate that the two drugs have approximately the same therapeutic and toxic potencies. This would agree with the comparative toxicity of the two substances as measured by the cat lethal dose assay. The mean (geometric) cat lethal dose is 0.116 mg/kg for ouabain(6) and 0.1866 mg/kg for 3-acetyl-strophanthidin(7).

Summary. In the failing heart-lung preparation of the dog, 3-acetyl-strophanthidin has been found to produce a positive inotropic effect comparable to that produced by ouabain.

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Esophagitis in Dogs Following Operations Employed in the Treatment of Mega-Esophagus.* (20566)

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The most severe complication resulting from the operative treatment of mega-esophagus, esophageal dystonia, or cardiospasm is chronic esophagitis. Indeed, this may become more disabling than the original condition which the operation was designed to correct. This study was devised to determine, in dogs, the incidence of esophagitis following the two standard surgical procedures for the treatment of mega-esophagus which have been most popular in recent years. These are the Heller operation and the Wendel operation, which are described in detail below.

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Materials and methods. Mongrel dogs weighing 8 to 16 kg were used in this study. Pentobarbital sodium was administered intravenously in a dosage of 30 mg per kg of body weight for anesthesia, and a mechanical respirator was used during surgery. The esophagogastric junction was exposed through an incision in the 10th left intercostal space, and where necessary the stomach and pyloroduodenal junction could be delivered easily into the chest by enlarging the esophageal hiatus. The Heller operation consists of an extramucous myotomy of the esophagogastric junction. In this study, the myotomy was made by incising the muscularis of the distal 4 to 5 cm of the esophagus and proximal 4 cm of the stomach longitudinally down to the submucosa with a sharp knife. Care was taken to leave no muscle fibers undivided. The Wendel procedure consists of a longi-

TABLE I. Esophagitis in Dogs Attending Performance of Heller and Wendel Operations on the Esophagogastric Junction. Degree of esophagitis as revealed by esophagoscopy, graded 0-4. Results are listed in 4 wk intervals.

Dog No.	Weeks 0–4	5–8	9–12	13–16	17–20	21–24	25–28	29–32	33–36
				Group 1. Heller operation					
1	0			0	1	1		1	1
2	0		0	0	1	1	1	1	1
3	0	0	1	0	0	0	0	0	0
4	0	1	0	1	0				
5	0	1	1	1					
6		0	0	0	0	1	0	0	
				Group 2. Wendel operation					
1		1	2	2		3	3	3	3
2		1	1	2	2	2	3	3	3
3	1	1	2	3	3				
4	1	1	2	3	3				
				Group 3. Heller operation + histamine					
1		2–3	3	3	3	3	4	(EP)*	
2		1	2	2	3	2	2		
3		2	2	3	3		3	3	
4		1	1	1	1	1	1	1	
				Group 4. Wendel operation + histamine					
1	1	2	2	3					
2	3	3	3	4	(EP)*				
3	1	2	2	3	3	3	3	3	
4	1	2	2	3	3	3	3	3	
				Group 5. Wendel operation and vagotomy + histamine					
1	1	2	3	3	3	3	3	3	
2	0	3	3	3					
3	0	1	2	2					
				Group 6. Wendel operation, vagotomy, and pyloroplasty + histamine					
1	0	0	0	1					
2	0	0	0	0					
3	0	0	0	0					
4	0	1	0	0					
5	0	1	1	3	(Isolated esophageal ulcer)				

* EP = Esophageal perforation.

tudinal incision through all layers of the esophagogastric junction which is closed transversely. In this study, the incision extended from 4 cm above to 4 cm below the esophagogastric junction. It was closed transversely in 2 layers—an inner mucosal stitch of continuous 000 plain catgut and an outer row of interrupted 0000 silk. After both procedures, the left crus of the diaphragm was carefully resutured to esophagus with a few interrupted silk sutures.

The animals were allowed to recover for 2 to 4 weeks following operation, and then esophagoscopy was performed every 2 to 3 weeks to assess the condition of their esophagi. Six groups of animals were studied. Group 1 animals had the Heller procedure alone, and Group 2 the Wendel operation alone. Groups 3 and 4 were similar to Groups 1 and 2 with the addition of daily intramuscular injections of 30 mg of histamine-base in beeswax. The dogs in Group 5 had a Wendel operation and in addition, a bilateral supradiaphragmatic vagotomy; and those in Group 6 had a Wendel operation, a vagotomy, and a Heineke-Mikulicz pyloroplasty. The animals in both Groups 5 and 6 were given daily injections of histamine-in-beeswax.

Results. The results are shown in Table I. When, at esophagoscopy, esophagitis was observed to follow these operative procedures, the process was found usually to be limited to the distal third of the esophagus; however, in some of the more severe cases, changes were present throughout the entire esophagus. The degree of esophagitis was graded on the basis of 0 to 4.

0—normal

1—mild esophagitis (slight to moderate hyperemia)

2—moderate esophagitis (more hyperemia, early linear erosions)
3—severe esophagitis (marked hyperemia, linear erosions which bleed easily, erosions covered by exudate)
4—esophageal perforation.

Of the dogs in Group 1 subjected to the Heller operation and not receiving histamine, none developed severe esophagitis. Contrast this with Group 2, in which all dogs developed severe esophagitis following the Wendel operation. The marked difference in the incidence of esophagitis between these 2 groups is probably the result of the difference in the degree of regurgitation of gastric juice into the lower esophagus. Some reflux of gastric juice was noted at esophagoscopy in dogs from both groups; however, this finding was more marked and more constant in the Wendel dogs.

Histamine-in-beeswax injections were given to produce a constant stimulus for the secretion of a large volume of highly acid gastric juice. It was believed that this would intensify any esophagitis following these operations inasmuch as regurgitation of acid peptic juice is the cause of esophagitis in these circumstances. In Groups 3 and 4, administration of histamine resulted in a severe degree of esophagitis in dogs with both the Heller and Wendel operations. One dog from each of these groups died following perforation of an esophageal ulcer.

The Wendel operation was chosen to test the effectiveness of vagotomy and pyloroplasty in preventing esophagitis because of the high incidence of severe esophagitis when the animals received histamine injections. In Group 5, vagotomy alone failed to prevent the development of severe esophagitis in dogs with the Wendel operation subjected to histamine stimulation. On the other hand in Group 6, vagotomy and pyloroplasty together were found to be effective in preventing esophagitis under the same circumstances. However, one of the animals in Group 6 did develop a small isolated esophageal ulcer on the posterior wall just above the esophagogastric junction with little or no evidence of esophagitis anywhere else in the distal esophagus. This ulcer was probably caused by constant exposure to the acid peptic secretion from a fold of gastric mucosa pulled upward in the transverse closure of the Wendel operation. It has previously been demonstrated that transplantation of a bit of fundic mucosa 1 cm in diameter pedicled on a source of blood supply into the wall of the esophagus is regularly followed by the development of a peptic ulcer in the esophageal mucosa in juxtaposition to the transplant.

The dogs of Group 6 in which a vagotomy and pyloroplasty were done, in addition to the Wendel procedure, remained in good condition and maintained their weight well. In contrast, the dogs in Group 5, a similar group in which a vagotomy was done but pyloroplasty was omitted fared badly. They all lost weight rapidly and soon became emaciated and died. The immediate cause of death was often pneumonia, but in many, cachexia was the only post mortem finding. In fact, many dogs were operated upon in this particular group before the 3 survivors for the experiment could be maintained alive throughout an adequate period of observation.

Summary. 1. Dogs subjected to the Wendel operation for mega-esophagus developed severe esophagitis, whereas dogs with the Heller operation did not. 2. Daily injections of histamine resulted in severe esophagitis in dogs with both the Heller and Wendel operations. 3. Vagotomy alone did not prevent development of esophagitis following the Wendel procedure when the animals received histamine. 4. Vagotomy and pyloroplasty together were effective in preventing esophagitis in dogs with the Wendel procedure receiving histamine injections.

ADDENDUM. Since this paper was submitted for publication, two new groups have been added. In Group 7, a Wendel operation and pyloroplasty were done, the dogs being given histamine; 4 of 5 such dogs developed severe esophagitis and one dog had no esophagitis. A 6th dog receiving no histamine developed a moderate esophagitis within 2 weeks following operation. In Group 8, a Heller operation, vagotomy, and pyloroplasty were done. The dogs also were given histamine; none of the 4 dogs developed esophagitis.

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Effect of Continuous Injection of Epinephrine on Adrenal Cortex and Anterior Hypophysis.* (20567)

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The role played by epinephrine in exciting the pituitary-adrenocortical axis during stress is not clear. Some investigators hold that this hormone acts only as a non-specific stressor. Others imply, however, that epinephrine is a specific "trigger" substance which activates the hypophysis to secrete corticotropin when the organism is under stress(1). McDermott *et al.*(2) postulate that epinephrine is involved particularly during the early phase of adjustment to stressful conditions. Most of the evidence in support of the latter position has been derived from short-term experiments. Since epinephrine disappears rapidly from the blood stream(3), its concentration cannot be maintained at a constant level if the hormone is administered by intermittent injections. The availability of an apparatus for continuous injection of small animals made possible this study of the effect of the prolonged action of epinephrine on the hypophysis and adrenal cortex. When so administered, one should expect to find histological evidence of accelerated secretory activity in these glands if epinephrine carries out a specific role in stimulating the release of corticotropin by the anterior hypophysis.

Procedure. Male rats of the Sprague-Dawley strain having an initial weight of approximately 300 g were used in these experiments. They were force-fed a medium carbohydrate diet(4) by stomach tube each morning and late afternoon. The technics and diet were modifications of those of Reinecke, Ball and Samuels(5). Each 24-hour dose of epinephrine hydrochloride (Upjohn) was contained in 1 ml of physiological saline with 1 mg of ascorbic acid added as an anti-oxidant (Groups 1-4, Table I). The control rats received an equal amount of saline and ascorbic acid. Several dilutions of epinephrine were made from the original 1:1000 concentration. In order to administer maximal doses of epinephrine over a period of weeks, it was necessary to begin with a moderate dose and to increase dosage as tolerance developed. Doses larger than those used here killed the rats. Each rat was placed in a metabolism cage which restricted its activity so that the animal was unable to reverse its position. A 21-gauge needle, having a barb attached to its shank to prevent withdrawal, was placed subcutaneously. Sterile needles were used for replacement every 48 hours. The solutions were administered to 6 rats simultaneously by a continuous injection machine. Three of the 6 rats received epinephrine and 3 received control injections. The room temperature was 74° to 78°F. At the end of a continuous injection period of 1 to 21 days the rats were anesthetized with cyclopal for autopsy.

Since Finerty, Hess and Binhammer(6) have postulated that accelerated secretion of corticotropin is accompanied by an increase in the phospholipid content of the anterior hypophysis, this material was stained in the pituitary glands of some animals by the method of Baker(7) after fixation in formaldehyde-calcium. Adrenal glands were fixed in Bouin's fluid and stained with the Masson(8) procedure. Others were preserved in 10% neutral formalin buffered to pH 7 by 0.2 M phosphate buffer. Frozen sections of these glands were stained with Sudan black B or by the Schultz(9) procedure for the demonstration of cholesterol and its esters. Paraffin-embedded sections of other adrenal glands similarly fixed were stained for esterase activity by the naphthol AS acetate technic of Gomori(10). The criteria used as evidence of secretion of corticotropin under the influence of epineph-

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TABLE I. Effect of Continuous Injection of Epinephrine on Mean Weight of Body, Thymus and Adrenal Gland.

Group	Treatment	Duration (days)	No. of rats	Body wt (g) Initial	Body wt (g) Final	Thymus wt (mg)	Adrenal (1) wt (mg)
I	Epinephrine 1:5000	7	9	273±11†	264± 8	121±66	30± 4
	1:2500	7					
	1:1000	7					
	Saline	21	9	278± 9	276±16	246±48	30± 2
II	Epinephrine 1:1000	2	3	296±10	295± 4		
	Saline	2	3	300±13	298± 5		
III	Epinephrine 1:5000	7	3	349± 9	313± 5		37±25
	1:2500	14					
	Saline	21	3	345± 9	303±18		29± 2
IV	Epinephrine 1:5000	1–2	4	326± 4	316± 4		
	Saline	1–2	4	333± 4	327± 5		
V	Epinephrine 1:2500	1	9	332±48		375±62	24± 2
	Saline	1	9	333±38		389±63	28± 3

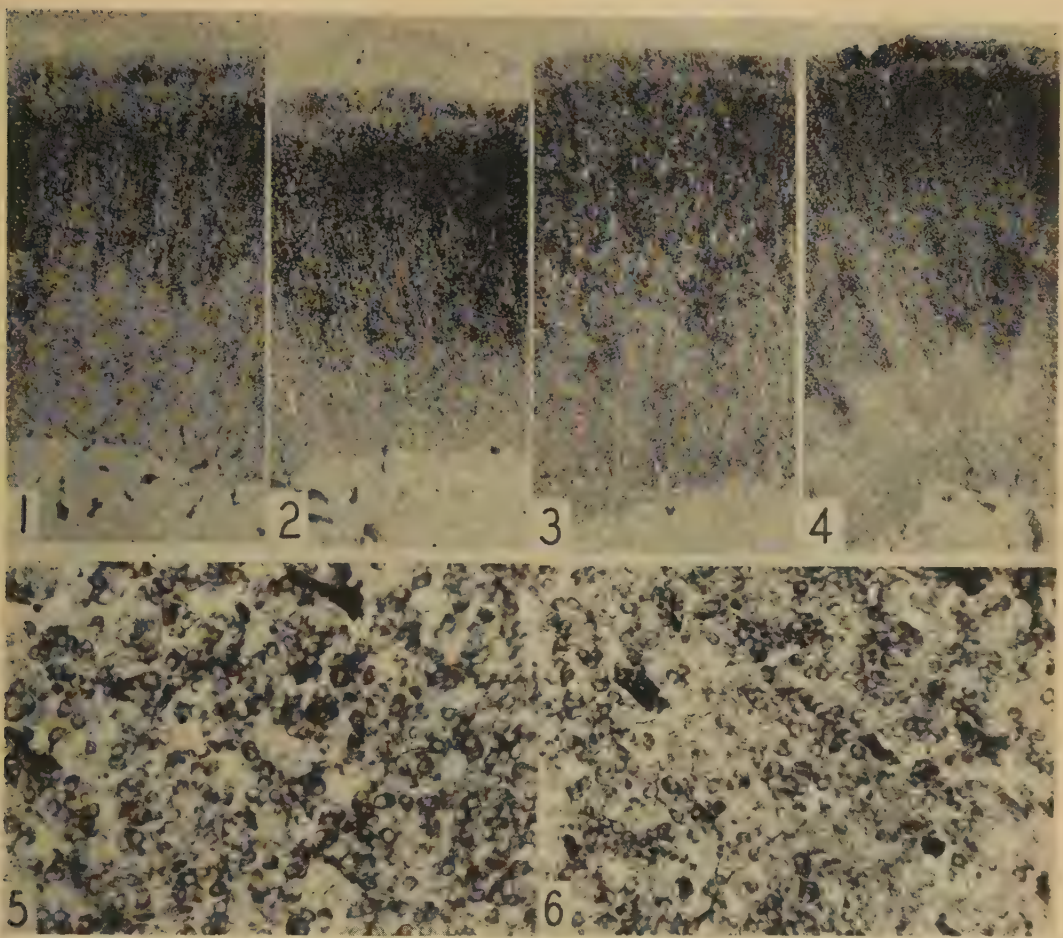
† Stand. dev. $= \frac{\sqrt{\Sigma d^2}}{N}$.

rine were: a) the phospholipid content of the anterior hypophysis, b) weight of the adrenal glands, c) size and mitotic activity of parenchymal cells in the zona fasciculata, and d) depletion of sudanophilic lipid and e) of cholesterol from the adrenal cortex. Since administration of exogenous corticotropin to rats elicits all of these changes in the adrenal cortex(11), epinephrine might induce similar effects if it is capable of causing the hypophysis to liberate significant quantities of corticotropin.

Observations. All of the rats exhibited transitory glycosuria which was maintained only when the dose of epinephrine was increased over that given at the beginning of treatment.

Fig. 5 and 6 show that the injection of epinephrine for 21 days did not increase the total amount of phospholipid in the anterior hypophysis or the number of cells which contained large amounts of phospholipid. As judged by all of the criteria of secretory activity used in this study, epinephrine failed to stimulate the adrenal cortex consistently. The total weight of the gland was not increased (Groups 1 and 5, Table I). An increase in mean weight of the adrenal glands is indicated in Group 3 but of the 3 rats receiving epinephrine, the adrenal glands of one were massive in size whereas those of the other two rats were lighter than the adrenal glands of their controls. Excluding this exceptional rat which exhibited signs of epinephrine toxicity and was near death prior to autopsy, there was no hypertrophy or hyperplasia of the parenchymal cells in the zona fasciculata of the cortex in any experiment. Significant depletion of sudanophilic lipid did not occur (Fig. 1 to 4). The intensity of color induced in the adrenal cortex by the Schultz procedure was somewhat less in only a few rats injected with epinephrine and ascorbic acid for 21 days. Esterase activity is evident in all zones of the cortex in control animals, being most intense in the zona glomerulosa and zona fasciculata. It was not altered in either zone by epinephrine.

Discussion. The auto-oxidation of epinephrine while in the hypodermic syringes presents a problem which may not have been solved completely in these experiments. It was prevented in the 21-day injection experiments by the addition of ascorbic acid to the fluid being injected. Bacchus *et al.* have reported that treatment of rats with ascorbic acid prevents the eosinopenia and depletion of adrenal cholesterol which occurs following the administration of epinephrine(12) but does not block the eosinopenic response which follows treatment with corticotropin(13). They concluded that ascorbic acid either antagonizes epinephrine directly or inhibits the release of



Adrenal cortices shown in Fig. 1 to 4 were fixed in 10% formalin and stained with Sudan black B (×60). Anterior hypophyses illustrated in Fig. 5 and 6 were stained with Baker's acid hematein (×210).

FIG. 1. Control for Fig. 2.
FIG. 2. After inj. of epinephrine (1:5000) for 48 hr without ascorbic acid.
FIG. 3. Control for Fig. 4.
FIG. 4. After inj. of epinephrine (1:5000, one wk; 1:2500, one wk; 1:1000, one wk) with ascorbic acid.
FIG. 5. Control for Fig. 6.
FIG. 6. After inj. of epinephrine as for Fig. 4.

corticotropin by the hypophysis. These observations indicate that ascorbic acid may have been responsible for the absence of pituitary-adrenal response to epinephrine in Groups 1 through 4. However, the quantity of ascorbic acid administered by Bacchus *et al.*(13) was enormous as compared with that used in our experiments. They injected 88 mg/100 g body weight intraperitoneally as compared with 1 mg per rat in our study. It seems improbable that this small amount of ascorbic acid could interfere with the action of epinephrine. Epinephrine did elicit the usual pharmacologic signs of its action in these experiments and in high dosage was definitely toxic.

In order to eliminate the use of ascorbic acid, epinephrine was injected continuously without ascorbic acid (Group 5, Table I) for 24 hours since during this period there was little evidence of oxidation of epinephrine as indicated by coloration of the solution. Likewise, in this experiment there was no histological evidence of adrenocortical stimulation by epinephrine.

As determined by chemical analysis, it is generally recognized that a fall in concentration of cholesterol occurs in the adrenal cortex immediately after administration of epinephrine for short periods of time(1). This effect is mediated by the increased secretion of corticotropin by the anterior hypophysis. The decrease in cholesterol which follows the injection of corticotropin involves the esterified but not the free form and may occur without any alteration in the amount of free fat in the adrenal cortex(14). The Schultz reaction applied to tissue sections is only a crude quantitative index of concentration and does not differentiate esterified from free cholesterol. In our experiments, a reduction in the former may have been masked by the free cholesterol present in the gland. Nevertheless, exogenous corticotropin induces a striking reduction in the intensity with which the Schultz procedure stains the adrenal cortex. The negative observations herein reported are important because they show that injection of epinephrine fails to duplicate the histological alterations induced in the adrenal cortex by injection of corticotropin(11) even though corticotropin presumably mediates the action of epinephrine on the adrenal cortex.

Several studies in man have failed to reveal significant stimulation of the adrenal cortex by the administration of epinephrine. Nelson *et al.*(15) found no rise in the concentration of 17-hydroxy adrenal steroids in the blood after intravenous injection of epinephrine. Jeffries *et al.*(16) did not observe an increase in urinary 17-ketosteroid excretion. Thorn *et al.* (17) reported that intravenous injection of epinephrine in quantities sufficient to cause eosinopenia and striking systemic reactions to the drug, failed to increase the output of 17-hydroxy adrenal steroids. Our observations concur with these investigations in man and lead to the conclusion that epinephrine is not capable of stimulating the hypophysis to secrete corticotropin at a rapid rate.

Summary. Under the conditions of our experiments, the continuous subcutaneous injection of massive doses of epinephrine into rats for 1 to 21 days, failed to elicit histological evidence of pituitary-adrenocortical stimulation.

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An Infusion Pump for Arterial Pressure Recording. (20568)

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The purpose of this communication is to describe an infusion pump which will make possible the continuous recording of blood pressure over long periods without an anticoagulant using high frequency recorders of the Hamilton, Sanborn, or Statham type. The principal requirements for such a pump are as follows: 1) it must deliver saline smoothly at a very slow constant rate into the artery from which the pressure recording is being taken; 2) it must not alter the magnitude of the pressure recorded but it must build up and sustain a pressure exceeding arterial by a small increment; 3) it must be provided with a device which will protect the artery and the pump in case of obstruction of the outlet because of a blood clot or for any other reason; 4) it must not introduce mechanical damping which would impair the frequency response of the manometer. The pump described below appears to meet these specifications.

In Fig. 1 is shown a schematic plan of the pump. The syringe holder and plunger drive assembly, which may be obtained commercially,* is mounted on any convenient base. It consists in a spur gear (not visible) which runs inside block A and drives rack B; this, in turn, drives a 50 cc standard syringe which is held in clamp C. It is necessary to ream the threaded hole in block A at the arrow to fit a piece of ¼″ drill rod D. This rod passes through the spur gear to which it is attached by a pin and terminates in a radio dial knob at E. The other end of the drill rod goes through a standard ¼″ radio panel bearing assembly in a vertical partition of masonite F, and terminates in spur gear G which meshes with spur gear H on the reduction box. Spring J enables one to disengage the spur gear in block A from rack B by pulling on knob E so as to quickly empty or refill the syringe by hand. The syringe may be emptied by 2 revolutions of knob E. In providing a drive mechanism a pair of Selsyn control transformers† have been inserted between the geared head motor and the reduction box in order to provide a variable torque slip clutch which will protect the syringe, gear train or blood vessel in case of obstruction of the outlet. These are connected in conventional fashion as indicated in Fig. 1 but are operated on 60 cycle current. When supplied with 15 volts from any suitable transformer they draw about 200 milliamperes. Under these conditions they will maintain exact synchronization and the pump will deliver saline against any pressure up to about 500 mm of mercury. When the pressure developed in the pump system exceeds this value, synchronization will break and the driven Selsyn will slip backwards and lock at a new angle of rotation. When this happens the pressure falls abruptly and then builds up again as before. This process will be repeated indefinitely until the high back pressure or obstruction is relieved. In Fig. 2 is shown the slow build-up of pressure and break of synchronization when the syringe outlet is purposely obstructed. Just before the slip occurs the current rises sharply which can serve as warning to the observer. Alternatively a relay operated buzzer or pilot light might be used for this purpose. A certain amount of adjustment in "slip" pressure may be obtained by varying the voltage applied to the Selsyn transformers. However, the electrical constants are not exactly reproducible because of inevitable variations in frictional resistance and small leaks.

It appears from the blood pressure tracing in Fig. 2 that neither the magnitude of the pressure recorded nor the frequency response of the manometer has been affected by introduction of the pump into the system. Probably flow occurs only during diastole but this has not been determined. For successful operation it is essential that leaks in the system be kept to a minimum since the flow rate

* Syringe Holder for Sodium Pentothal Anesthesia, Foregger Co., New York.

† General Electric Model No. 2J1G1.

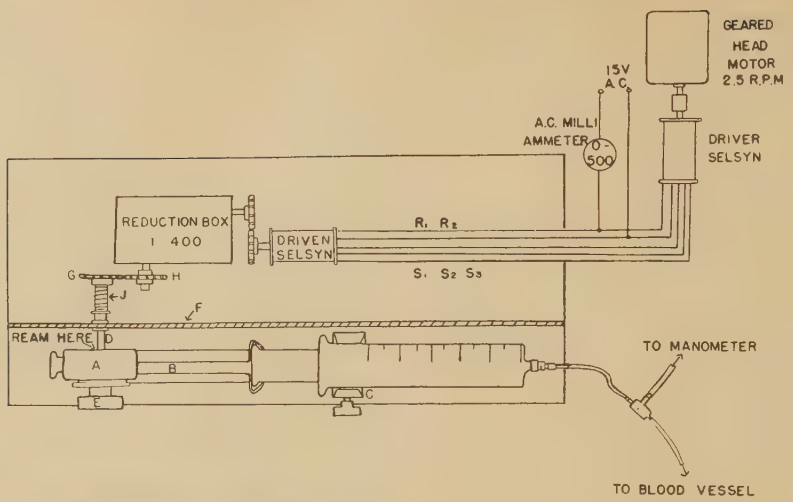

FIG. 1. Schematic plan of infusion pump. Explanation in text.

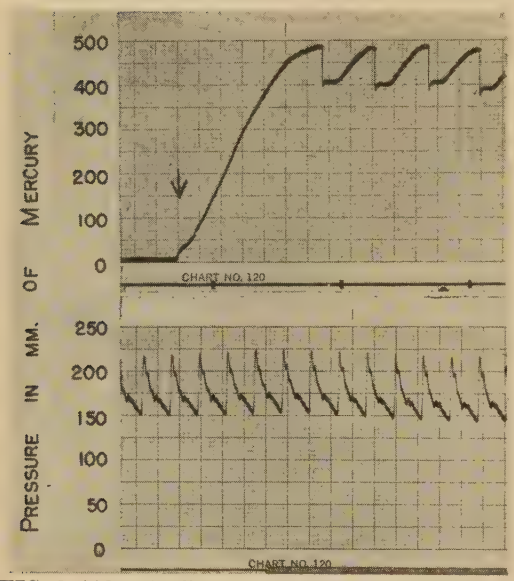

FIG. 2 (Above). Pressure recording with the outflow of the pump obstructed at the arrow. Time, 20 sec/cm. (Below) Blood pressure in femoral artery of an anesthetized cat. Time, 0.4 sec/cm. The pump was connected to the system at the signal. Note that no alteration of the high frequency components of the pressure waves occurs.

is very small and the saline will follow the course of least resistance. The syringe must be lubricated to prevent back seepage between the barrel and the wall. For this purpose a wettable grease is necessary to avoid trapping of air bubbles and polyethylene glycol 1500 has been found to be satisfactory. Luer-Lok fittings to polyethylene tubing were used for connection to animal and manometer. Luer stopcocks frequently caused leakage and were avoided.

As set up in Fig. 1, the delivery of the syringe will be:

$$\frac{(2.5 \text{ r.p.m.}) \times (25 \text{ cc/rev. of spur gear in block A})}{400} \times 60 = 9.4 \text{ cc/hr.}$$

This rate has been found to be sufficient to prevent clotting without heparin while recording blood pressure with the Sanborn Electromanometer continuously for 5 to 6 hours. In order to increase the delivery rate at the same "slip" pressure a faster motor may be used. For intravenous infusion at a high rate and low "slip" pressure the reduction box may have a smaller ratio. Exact control of the volume of infusion fluid is possible if a synchronous motor is used to rotate the driven Selsyn. Any convenient gear shift mechanism could be added to increase flexibility.

Although several infusion pumps are available commercially‡ it was felt that the pump described above might be of interest because of the protective mechanism, the freedom from damping and because it can be assembled from relatively inexpensive standard components with a negligible amount of shop work.

‡ Phipps and Bird, Richmond, Va.; Sanborn Co., Cambridge, Mass.; Metro Industries, L. I. City, N. Y.

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Factors Influencing Development of Insulin-Induced Hypoglycemic Convulsions in the Rabbit. (20569)

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The introduction of the rabbit as a test animal for insulin assay was followed shortly thereafter by the recognition of the unreliability of convulsions in this species as a criterion of insulin potency(1,2). Nevertheless the rabbit has been considered satisfactory for the demonstration of insulin-induced hypoglycemic convulsions in many teaching laboratories. For the last 5 years, however, administration of "convulsive" doses of insulin in this laboratory failed to elicit seizures. Examination of possible changes in the origin and handling of rabbits in recent years yielded no definite clues to the cause of the extreme difficulty encountered. The source, breed, and diet of the animals had not been changed. That the problem was not merely a local one has been indicated by communications with workers in other parts of the country. Several reports in the literature have related variations in insulin sensitivity to breed(3), color (4), age(4), size(1) and sex(5). Acton and Bose(3) found a much higher maximal blood glucose level after epinephrine administration in resistant groups and postulated that the increased protection against the effects of insulin was due to a higher compensatory epinephrine output. Despite postulates by several workers(1,6) that differences in sensitivity were due to variations in liver glycogen, this had not been investigated in any systematic fashion. The following series of experiments was undertaken to study these aspects of the problem.

Methods. Twelve rabbits, unselected as to breed and sex and weighing approximately 2 kg initially, were used in these experiments. The animals were placed on the standard laboratory diet of rabbit pellets supplemented with fresh lettuce for 2 weeks before the experiments were started. Four procedures were carried out on each animal: 1) subcutaneous injection of regular insulin (Iletin, Lilly), 2 units/kg; 2) a repetition of this; 3) subcutaneous injection of 1:1000 epinephrine, 0.25 ml(3); 4) terminal determination of liver glycogen immediately after induction of anesthesia by nembutal injected intravenously. An interval of at least a week from the previous experiment and a 24-hour fast preceded each procedure. By keeping conditions similar in this fashion and by starting each procedure at the same time of day, it was believed that errors due to diurnal variations, etc. could be minimized. Blood samples were taken for glucose analyses before insulin or epinephrine administration and at hourly intervals thereafter. When hypoglycemic convulsions occurred, they were rapidly terminated by intravenous injection of 2 ml of 50% glucose. Glucose determinations, by the micro-method of Somogyi-Shaffer-Hartmann(7), were run on protein-free blood filtrates prepared by the method of Somogyi(8). That the glucose values obtained by this method represent true sugar was demonstrated by determining the difference in reducing substances in filtrates of rabbit blood before and after yeast fermentations. Glycogen was determined by the method of Good *et al.*(9) with corrections made for non-fermentable reducing substances.

Results and discussion. The data obtained in the above experiments divided the animals into two groups, those which exhibited convulsions at one time or another after insulin administration, the reactors, and those which did not, the non-reactors. Only 7 of the 12 rabbits treated with so-called convulsive doses of insulin responded with convulsions. Table I presents a summary of the investigation of various metabolic factors of possible significance in this problem.

Contrary to expectation and to postulates in the literature, the reactors and the non-reactors had almost identical liver glycogen values. It should be pointed out that glycogen was determined at a time of day comparable to that of the initiation of the insulin

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TABLE I. Metabolic Factors of Possible Significance in Causation of Hypoglycemic Convulsions.

	Initial wt, kg*	Insulin I — Min glycemia, mg %	Insulin I — % of original	Insulin II — Min glycemia, mg %†	Insulin II — % of original†	Epinephrine — Max glycemia, mg %	Epinephrine — % of increase	Liver glycogen, terminal, g %
Reactors:								
# observations	7	7	7	6	6	7	7	7
Mean value	2.1	17.6	22.4	6.0	7.3	196.0	169.6	.31
Stand. error	.1	4.0	5.0	1.5	1.6	11.4	18.3	.14
Non-reactors:								
# observations	5	5	5	5	5	5	5	5
Mean value	2.8	27.8	37.6	29.6	35.2	195.2	174.0	.33
Stand. error	.2	8.5	13.3	5.0	4.2	6.1	33.5	.15

* Significant difference between groups, $P < .02$.
† Highly significant difference between groups, $P < .001$.

tests, and that the values represented, as nearly as possible, the stores upon which the animal could draw when hypoglycemia developed. That factors other than those described by Acton and Bose(3) were operating in the present series was indicated also by the results of epinephrine administration, which might be considered an *in vivo* measure of liver glycogen or its availability. The values summarized in the table were examined not only by statistical comparison of the means of the two groups but also in terms of possible relationships to body weight, initial blood glucose level and, in the case of epinephrine, dosage. No correlations were found. Although liver glycogen constituted a major carbohydrate reserve in the protection of the animal against hypoglycemia, it had little to do with whether convulsions occurred when hypoglycemia developed.

By definition, a low blood glucose level is a prerequisite for exhibition of hypoglycemic symptoms. It is perhaps surprising that no significant differences in the extent of hypoglycemia were apparent between the rabbits which exhibited convulsions in the first insulin test and those which did not. All animals were hypoglycemic. An increase in insulin dosage would have affected the duration rather than the depth of hypoglycemia(10). Contrary to the observation of Dotti(11) that fermentable reducing substances were absent from the blood during hypoglycemic convulsions, values of 7 and 18 mg % were found during such seizures on 2 separate occasions. However, at the time the samples were taken, convulsions *per se* had constituted an adequate stress to bring into play compensatory mechanisms; the glucose level which might have constituted the stimulus to convulsions was not assayed. One animal was outstanding in its ability to withstand glucose levels of 0 to 11 mg % for prolonged periods before convulsing. Convulsions were elicited only when the animals were hypoglycemic, but the absolute glycemic levels did not appear to determine their appearance(2).

Administration of a second dose of insulin, in most instances one week later, revealed an unexpected change in sensitivity in the reactor group. Although there were no significant differences between values obtained in the first and second tests in the non-reactor group, the reactors exhibited a marked increase in sensitivity with significant differences obtained between Insulin I and Insulin II. In each individual case not only did the blood glucose fall to a lower level but it also fell more rapidly than in the first insulin test. This was not true of the non-reactors. The increased sensitivity observed in these experiments was probably related to the phenomenon of "education" to insulin reported by Sahyun and Blatherwick(4), the observation that rabbits treated with convulsive doses of insulin at weekly intervals required lower doses of the hormone to produce seizures. The present series of experiments afforded no explanation of the phenomenon.

This increase in sensitivity produced a serious problem in the interpretation of the results of the second insulin test. Although the same factors were assayed in Insulin II as in Insulin I, highly significant differences between reactors and non-reactors were demonstrated

statistically, because the change in sensitivity had occurred in only one group. In view of the negative results of the first insulin test, however, one must conclude that the glycemic level did not become the primary determining factor in the causation of convulsions when the test was repeated.

The time at which convulsions occurred constituted another interesting aspect of the insulin tests. In 9 cases (5 rabbits) seizures took place within the 4 hours considered to be the period of maximal action of the hormone. In only 2 instances was the response delayed. One of the late reactors was the only animal which changed categories in the course of the experiments; although it did not react to the first dose of insulin, a week later a similar dose caused hypoglycemic convulsions in 6.5 hours. This might be another manifestation of increased insulin sensitivity. The other late reactor did not convulse until 7 hours after the first insulin administration, although its blood glucose level had remained below 11 mg % for 4 hours prior to this.

Several other comparisons between reacting and non-reacting animals were made besides those included in Table I. Analyses of initial blood glucose levels in the two groups revealed no significant differences; the mean value for all experiments was 76.7 mg % with a standard error of 2.0. There appeared to be no definite variation due to color or breed in these experiments; *i.e.*, 4 Chinchillas, 2 New Zealand albinos and 1 Dutch rabbit were reactors, 3 New Zealand albinos, 1 Chinchilla and 1 Himalayan were non-reactors. This observation constituted a difference from the experimental results of Acton and Bose(3) and a similarity to results reported in this country by Dotti(5). The fact that each of the 3 females studied exhibited convulsions can hardly be called significant or a confirmation of Dotti's reported sex difference in view of the small number of females involved in the present experiments. There was no observable difference in physical condition of the two groups. Coccidial nodules were present in the livers of 4 reactors and of 3 non-reactors; however none of the animals had shown other signs of the infestation.

The only other observation which had bearing on the problem was the difference in weights of the animals. Intergroup comparisons of the means of initial and terminal weights yielded P values less than 0.02 and 0.05, respectively. If these were significant differences, this observation might afford partial explanation of the difficulties encountered in our teaching laboratory. The animals used in the demonstrations were usually large rabbits which had been in the animal quarters for some time.

On the other hand, it must be admitted that none of the observations made in the course of this study fully explained why half of the present series of animals developed convulsions after insulin administration and the other half failed to show symptoms although their blood glucose levels were just as low in the first insulin test. As mentioned earlier, clearly more was involved in the elicitation of convulsions than the blood glucose level and/or its rate of fall.

The observation that the categories established in the first insulin test, reactors and non-reactors, were borne out in the second insulin test, that with only the one exception each rabbit remained in the same group, has led to the conclusion that similar factors must have been operating in both tests to determine whether a given rabbit reacted or not. Results of the experiments indicate that the factors investigated did not determine into which category each animal fell. In view of recent studies of humans responding to hypoglycemia with convulsive seizures(12), one is tempted to question whether the differences in reactivity may be a characteristic of the central nervous system and its excitability rather than a characteristic of only the metabolic-hormonal pattern of the individual. This aspect of the problem awaits investigation.

Summary. Of a group of 12 rabbits treated with "convulsive" doses of insulin, only 7 responded with convulsions. Except for the occurrence of convulsions, the reactors could not be distinguished from the non-reactors in the glycemic response to the first insulin injection, in response to epinephrine administration, or in liver glycogen. Significant differences were obtained in body weights and in the glycemic response to the second in-

sulin injection. The latter was related to an unexplained increase in insulin sensitivity in the reactors. No change in sensitivity occurred in the non-reactors.

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Botulinum Toxin and the Motor End Plate.* (20570)

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The extreme toxicity of botulinum toxin has long been known to be due to its paralytic action on skeletal muscle. Guyton and MacDonald(1) confirmed and extended the observation that peripheral nerve conduction in toxin-paralyzed rabbits and guinea pigs was unimpaired. They also pointed out that the skeletal muscles of such organisms could contract if stimulated directly by electric shock. It seemed evident, therefore, that the toxin exerts its effect upon the neuromuscular junction by blocking conduction (a) in the terminal fibers or (b) in the motor end plate (perhaps by inhibiting the release of acetyl choline from the end plate). Burgen, Dickens, and Zatman(2) demonstrated that the amount of acetyl choline released by a nearly toxin-paralyzed muscle was greatly decreased; it was less than 15% of the original output. Brooks(3), using cat and guinea pig muscle, has presented preliminary evidence in favor of the blocking hypothesis (a). The present investigation was undertaken in the hope of adducing more evidence for the site of action of the toxin.

Materials and methods. The preparation used was the sartorius-sartorius nerve of the frog, *Rana pipiens*. The muscle was mounted in a lucite chamber and bathed with Frog Ringers. A water jacket maintained the temperature at 25°C. The muscle was stimulated indirectly via the sartorius nerve with short (0.1-0.3 msec) pulses of 1.5 V amplitude obtained from a Grass S4A stimulator. The electric reactions of the muscle were detected with a platinum-iridium, glass-coated microelectrode, led off through a single stage cathode follower amplifier mounted directly on the electrode. The signal was then carried through a differential preamplifier with voltage gain of 1000 and pass band of 0.2-40 KC and thence to a DC coupled amplifier with pass band of 0-100 KC. The signal was displayed on one gun of a 3-gun CRO. Time signals were injected on the trace of another gun, operated from a common sweep generator. The time signal generator was calibrated against a crystal controlled pulse generator. End plate potentials were located by probing when the muscle was nearly paralyzed with toxin or by poisoning the muscle with a 1 μM solution of curare

* The opinions or assertions contained herein are the private ones of the writers and are not to be construed as official or reflecting the views of the Navy Department or the naval service at large.

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and moving the microelectrode until an end plate was found. After the muscle recovered from curare, type A botulinum toxin was applied, the most convenient concentration being 1.25 x 10^6 mouse LD_{50}/ml; this concentration poisoned the muscle in 3-4 hours. In several experiments check assays of the diluted stock toxin solution were made on mice.

Results. For a period of about 15 minutes after botulinum poisoning becomes evident (failure of a previously supramaximal stimulus to elicit a muscle action potential) 2 such stimuli delivered in close succession will cause a muscle spike (and contraction). This is the familiar summation of subliminal stimuli. If the toxin is paralyzing by blocking conduction in the terminal nerve fibers, then summation may be explained by the first stimulus electrotonically increasing the excitability of the nerve on the distal side of the block and the second stimulus "jumping" the block, which results in a contraction of the muscle(4). It is obvious that only the stimulus artifact would be recorded in response to the first stimulus and in response to the second, a stimulus artifact followed by a muscle action potential. If, however, the action of the toxin is to depress the end plate mechanism, one might expect to observe a small end plate potential (insufficient to trigger the muscle spike) after the first stimulus. Summation could then be explained in terms of the acetyl choline theory. The first stimulus causes release of a subthreshold quantity of acetyl choline which adds to that released by the second stimulus and contraction follows.

Our observations lend support to the second hypothesis. In 6 experiments we consistently observed end plate potentials following the first "unsuccessful" stimulus (Fig. 1a). If a second identical stimulus is applied within 4-18 msec, summation occurs and a muscle action potential is observed (Fig. 1b). If the 2 stimuli are of the order of 20 msec apart, no summation occurs and 2 end plate potentials are recorded (Fig. 1c). It is possible of course that the terminal fibrils are poisoned secondarily, after the end plates have been poisoned.

Burgen, Dickens, and Zatman(2) also have demonstrated that botulinum toxin affects

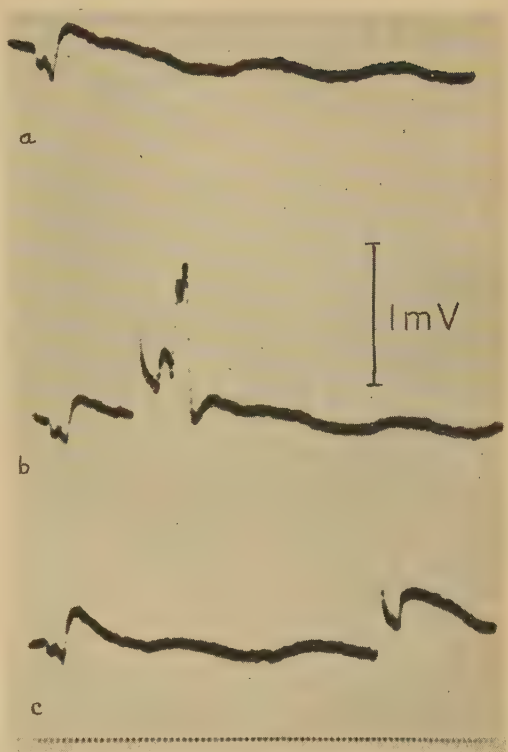

FIG. 1. Response of a partially paralyzed muscle to electric stimuli. (a) An end plate potential in response to a single stimulus. (b) Summation of end plate potentials in response to 2 stimuli 11 msec apart. (c) Two end plate potentials recorded in response to 2 stimuli 32 msec apart. Time: 0.28 msec marks.

neither the enzymes acetylating choline nor choline esterase and that added acetyl choline will cause a toxin-paralyzed muscle to contract. It is most probable, therefore, that the paralytic effect of the toxin is due to poisoning of the mechanism which releases acetyl choline.

Summary. The paralytic action of botulinum toxin is due primarily to a depression of the motor end plate mechanism and not to a blocking of the terminal nerve fibers. The mechanism, whereby acetyl choline is released, is probably inhibited.

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A Colorimetric Method for Determination of Vitamin A and Carotene by Perchloric Acid.* (20571)

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In the course of studies of the mode of action of vit. A(1), it was noted that the unstable color which vit. A develops in the presence of many inorganic acids(2) often could be made more stable in a solution of amyl acetate. Perchloric acid was found to give the most intense color with vit. A and with carotene. On this basis a colorimetric method was devised, which makes possible the quantitative estimation of vit. A and of carotene in each other's presence.

Reagents. 1. Vit. A standard solution. Crystalline vit. A alcohol (Eastman-Kodak) or synthetic vit. A palmitate in corn oil (Hoffmann-LaRoche)† freshly dissolved in chloroform to a final concentration of 300 μg/cc and serially diluted. A standard curve is prepared with 0.1 ml volumes of these solutions, corresponding to 3-30 μg of vit. A. 2. Carotene standard solution. Carotene freshly dissolved in chloroform to final concentrations of 30-300 μg/ml. The standard curve is prepared with 0.1 ml amounts of these solutions. 3. Amyl acetate. 4. Concentrated hydrochloric acid. 5. Perchloric acid reagent. Three volumes of a concentrated solution of perchloric acid (70-72% perchloric acid) diluted with one part of distilled water. After cooling, the reagent is stored at ice box temperature. 6. Absolute alcohol.

Procedure. 1. Preparation of standard curves. Samples of 0.1 ml of the standard vit. A (3-30 μg) or carotene (3-30 μg) solutions are measured into test tubes and diluted with 1.6 ml amyl acetate. To this mixture 0.4 ml concentrated hydrochloric acid is added. The tubes are shaken and allowed to stand at room temperature for 4 to 5 minutes. This is followed by addition of 2 ml perchloric acid reagent. Carotene gives a bluish-green color with a broad maximum absorption between 725 and 760 mμ. With vit. A an immediate blue color develops which almost instantaneously changes to a purplish red tint with a maximum absorption between 520 and 530 mμ. The contents of the tubes are mixed with 0.3 or 0.4 ml absolute alcohol with vigorous shaking until the layers become miscible. The purplish-red color of vit. A, although losing in intensity, is visible for 25-30 minutes, after which it gradually turns yellow; the bluish-green color of carotene is somewhat more stable. The color was measured at the same time, 5 to 15 minutes after addition of the perchloric acid reagent. A mixture, containing 0.1 ml of chloroform in place of the vit. A or carotene solutions, prepared simultaneously with the other standard mixtures, serves as a blank. The blank mixture gradually turns yellowish on standing. Three standard curves are drawn: Vit. A is measured at 525 mμ, carotene at 525 and 750 mμ (Fig. 1). The curves follow the Beer-law in the above mentioned concentrations.

2. Estimation of unknown amounts of vit. A and carotene. A sample is diluted with chloroform to the proper range in which determinations are to be carried out. The subsequent steps in the color development are the same as those for the standard solutions.

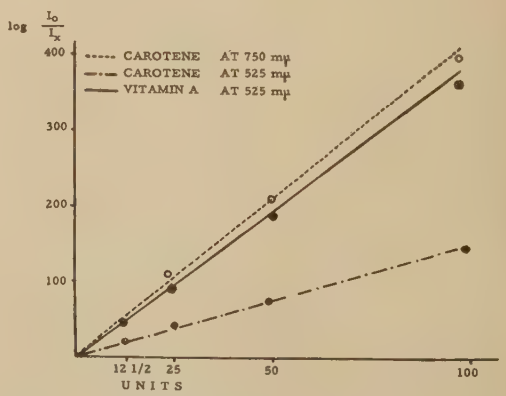

FIG. 1. Absorption curves of vit. A and carotene with perchloric acid.

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† Obtained through courtesy of Dr. S. Evert Svenson, Hoffmann-LaRoche, Inc.

When only carotene was present, the readings were made at 750 mμ. When the unknown contained vit. A only, readings were made at 525 mμ and the amounts read from the standard curve for vit. A. In this case, determinations may be also carried out with the Klett-Summerson photoelectric colorimeter with filter No. 54. When both carotene and vit. A were present in the unknown, the following procedure was adopted: Readings were made at 525 mμ and 750 mμ. At 750 mμ the absorption of vit. A is negligible and readings made at this wave length permit the direct quantitative estimation of the carotene present in the unknown sample. The reading which corresponds to these amounts of carotene at 525 mμ is determined from the standard carotene curve (525 mμ) and subtracted from the reading obtained for the unknown at 525 mμ. The resulting figure then is read from the vit. A standard curve and gives the amounts of vit. A in the unknown sample. Comparison with Sobel's method(3) gave excellent agreement for vit. A in samples of fish liver oil. When 25 U of vit. A was added to a sample of fish liver oil containing 33 to 34 units, the recovered amounts were 58 to 60 units. Vit. D gave no color with the perchloric acid reagent.

Discussion. The color developed by vit. A in the presence of inorganic acids or some salts of strong acids (such as $SbCl_3$) is believed to be due to the formation of a colored vit. A cation(2,4). In the course of this cation formation, the absorption of vit. A shifts from the ultraviolet region to longer wave lengths. The first color shift to the blue, as occurs in the Carr-Price method, in the first stage of Sobel's method or in the present method, appears to be always very unstable. Only when a further shift to the purple region occurs, as in Sobel's or the present method, is there a fair degree of stability of color.

Among the advantages of the present method is the ready availability and stability of the inexpensive reagents which may be of value in large scale determinations. The color developed with perchloric acid follows the Beer-law over wider ranges than the colors obtained with previous methods. One of the main drawbacks of the method is its relatively low sensitivity, as compared with previous methods. The slow, but continuous fading of the developed color may lead to a lesser degree of accuracy than that obtainable with other methods, unless readings are made at exactly the same time after addition of the perchloric acid and under the same conditions.

Summary. A method is described for the quantitative colorimetric estimation of vit. A and carotene in each other's presence. The method is based on the fact that the purple-red color developed by vit. A (maximum absorption 525-530 mμ) and the bluish-green color developed by carotene (maximum absorption 725-760 mμ) in the presence of perchloric acid, may be stabilized in a solution of amyl acetate. In a mixture of vit. A and carotene, carotene may be estimated directly by determining its absorption at 750 mμ; the amounts of vit. A may be calculated by subtracting from the absorption, measured at 525 mμ, the absorption value which corresponds to carotene at this wave length.

The technical assistance of Miss Sondra Golomb is acknowledged.

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Effect of Dermal Contact with Cold on the Coronary Circulation.* (20572)

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It is well-known that patients with arteriosclerotic heart disease and angina pectoris frequently experience precordial pain upon exposure to cold. The interpretation given this observation is that dermal contact with cold elicits a reflex constriction of the coronary arteries, and that the resulting myocardial ischemia is responsible for the chest pain and electrocardiographic changes(1,2). The existence of such reflexes has not been established. The following study was initiated to determine the presence or absence of such reflex effects of cold on coronary blood flow (CBF) in the dog.

Methods. Experiments were performed on pentobarbitalized open-chested dogs with respiration maintained artificially. A small segment of the circumflex branch of the left coronary artery was dissected free, cannulated near its origin, and perfused via the subclavian artery. Care was taken to maintain the integrity of the sheath of the artery. A blood reservoir and pump perfusion system were placed in parallel with the arterial perfusion system in order to permit a selection of perfusion pressures, regardless of the dog's mean aortic pressure(3). Coronary inflow was measured by an optically recording rotameter(4). Aortic and mean coronary inflow pressures were registered by modified Gregg manometers. Temperatures, recorded from the right atrium and the coronary inflow cannula, remained constant throughout the experiments.

Results. In 5 out of 6 preliminary experiments in which ice was placed in contact with one hind limb, no change in CBF, heart rate or arterial blood pressure was detected. In the 6th experiment, CBF, aortic pressure, and heart rate increased while coronary perfusion pressure was held constant. In 10 subsequent experiments the strength of the cold stimulus was increased by immersing 1/3 to 2/3 of the body surface in ice water. In all these experiments, contact with cold in the absence of changes in heart blood temperature produced an increase in CBF which was associated with an increase in aortic pressure and/or heart rate. Fig. 1 is a plot of a representative experiment in which CBF, perfusion pressure, aortic pressure, and heart rate are plotted against time. Following immersion in ice water, CBF increased, and was associated with an increase in mean aortic pressure and pulse pressure. Adjustment of coronary perfusion pressure to control levels at 5½ and 8½ minutes resulted in a moderate reduction in CBF, but not to pre-immersion values. Heart rate showed no significant change in this experiment. In Table I are presented data on all 10 experiments. In the first 5 experiments perfusion pressure was kept constant, yet CBF increased concomitantly with mean blood pressure and pulse pressure. In the last 5 experiments in Table I, either no attempt was made to regulate perfusion pressure or, for technical reasons perfusion pressure could not be held at control levels. Therefore, part of the increase in CBF in these experiments may be attributed to the

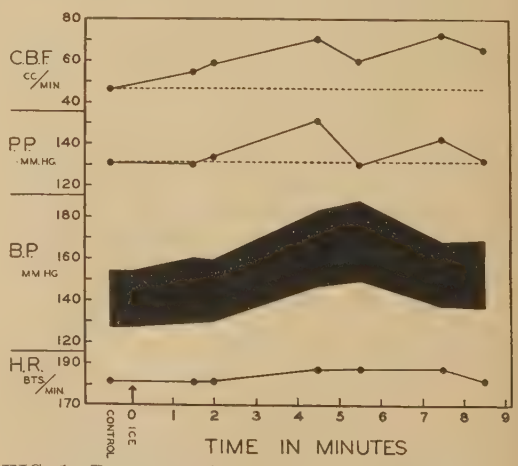

FIG. 1. Representative experiment illustrating the effect of partial immersion in ice water on coronary blood flow (CBF), perfusion pressure (PP), arterial blood pressure (BP), and heart rate (HR).

* This investigation was supported by a grant from the Life Insurance Medical Research Fund.

TABLE I. Cardiovascular Effect of Dermal Contact with Cold, 10 Experiments.

	Aortic pressure, mm/Hg	Heart rate, beats/min.	Perfusion pressure, mm/Hg	CBF, cc/min.
Cont.	145/121	150	121	38
Exp.	158/130	158	122	45
Cont.	140/120	214	128	32
Exp.	156/125	214	130	51
Cont.	145/126	160	124	46
Exp.	170/147	154	124	57
Cont.	154/127	182	131	46
Exp.	187/149	187	129	59
Cont.	134/119	158	126	35
Exp.	146/126	171	128	48
Cont.	116/ 87	187	98	39
Exp.	123/ 95	193	103	48
Cont.	140/119	158	123	45
Exp.	177/147	194	145	100
Cont.	141/126	200	129	48
Exp.	161/138	200	140	66
Cont.	147/113	158	125	59
Exp.	162/122	157	132	65
Cont.	134/103	154	117	31
Exp.	148/117	158	129	35

rise in perfusion pressure.

It should be noted that in 8 experiments pulse pressure increased, whereas heart rate was significantly altered in only 2 experiments. Stimulation of the accelerator nerves following coronary artery cannulation produced an immediate increase in CBF in the cannulated vessel.

Discussion. The increase in CBF observed upon immersion of the anesthetized dogs in ice water was associated with an increase in cardiac work as judged from the increase in aortic pressure, pulse pressure, and/or heart rate. With less intense stimulation (contact of hind leg with ice), CBF was unchanged, but cardiac work was also unchanged. In no instance did CBF decrease. Therefore, no evidence could be uncovered for reflex coronary constriction and the increase in CBF observed appeared to be related to the increase in cardiac work.

Criticism may be leveled at these experiments because of the depressant effect of anesthesia upon reflexes and the possible severance of sympathetic nerves in cannulation of the coronary artery. However, anesthesia was light; tendon and corneal reflexes were active; autonomic reflexes were also active, as evidenced by the reflex rise in blood pressure and increase in heart rate upon contact with ice water. That sympathetic nerves were intact is suggested by the prompt increase in CBF obtained upon stimulation of the accelerator nerves.

Our results are at variance with those of Gilbert *et al.*(5) who reported small decreases in CBF upon irrigation of the nasal mucous membranes with ice water. These investigators used the thermostromuhr and coronary sinus outflow technic to measure CBF in the decerebrate dog. Gregg(6) has pointed out that the thermostromuhr and the coronary sinus outflow technic are not accurate enough to attach significance to changes of the order of magnitude observed by Gilbert and coworkers.

Application of our data to man must be made with caution. However, our findings offer no support for the clinical interpretation that chest pain and ECG changes appearing upon dermal contact with cold in patients with angina pectoris are due to reflex coronary artery constriction. The possibility of the failure of the sclerosed vessels to dilate in response to an increase in cardiac work has not been excluded.

Summary. Dermal contact with ice water in the anesthetized dog failed to produce a reflex constriction of the coronary arteries. Exposure of small areas of body surface to ice water resulted in no changes in CBF, whereas immersion of 1/3 to 2/3 of the body surface in ice water produced an increase in CBF associated with an increase in cardiac work.

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Relation of Thyroid Status to Nitrogen Excretion Following Exposure to Thermal Radiation.* (20573)

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(Introduced by M. John Boyd.)

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The well known negative nitrogen balance which follows severe burning has not yet been fully explained. The conclusion that it is a complex phenomenon, not related to any single biochemical event, appears to be justifiable when the reports of various investigators are considered(1-4). The increased excretion of steroids which accompanies the period of greatest nitrogen excretion(5,6) and observations relative to the thyroid(7,8) imply abnormal endocrine functions during the post-burn state. Endocrine involvement is to be expected in view of the alarm and stress reactions(9) which the organism undergoes with thermal trauma. Our experience that thyroid activity (as measured by radio-iodide uptake) of burned animals deviates from normal(10), and the study of Gribble and Peters(8) which suggested the increased nitrogen excretion to be related to altered thyroid status, demanded that these biochemical parameters be measured in intact animals with normal, hypo- and hyperthyroid status.

If the activity of the thyroid is related to the increased nitrogen excretion which follows thermal assault, then changes in nitrogen output in hypo- and hyperthyroid burned rats should differ from that of euthyroid burned animals. Euthyroid nitrogen excretion (post-burn) should approach that of the hyperthyroid rats, whereas nitrogen output of the hypothyroid beasts should show little change. The following study was made to test this hypothesis.

Experimental. Three groups of adult male rats, twelve rats per group, were selected for uniform body weight (190-210 g) and fed an 18% casein ration(11). One group received 0.5% desiccated thyroid (Armour) in the basal diet. The second group received 0.5% thiouracil, whereas the third group received no additions and served as controls. Paired feeding was employed throughout with the thiouracil-fed rats acting as the limiting group from the beginning. Three-day nitrogen balances were measured starting on the 21st day of these dietary regimes and on the 28th day. By this time all 3 groups were in comparable states of negative nitrogen balance as indicated in Table I. On the 34th day half of the animals in each group were shaved and burned by one minute exposure of 20% of the total body surface (midriff) to dry heat emitted from an array of six 150 watt electric lamps concentrically arranged. The caloric flux of this device has been estimated to be the order of 14-15 g cal per cm^2 per minute. After burning, a third 3-day nitrogen balance was then measured. Thyroid status of the 3 unburned groups was evaluated by I^{131} uptake at the end of the experiment. Methods of radioassay have been described elsewhere(10).

Results. As indicated in Table I, burned hyperthyroid rats excreted 50% more urinary nitrogen than did unburned hyperthyroid animals. Total nitrogen excretion of the burned rats of this group increased 19%, nitrogen bal-

TABLE I. Nitrogen Metabolism in Relation to Thyroid Status Pre- and Post Burning.
(All values as mg N/100 g body wt.)

Group	Period, day	Fecal N.	Urinary N.	N. balance
Hyperthyroid	21st–24th	588	577	–1089
Euthyroid	"	626	462	–1020
Hypothyroid	"	454	358	– 749
Hyperthyroid	28th–31st	399	173	– 536
Euthyroid	"	339	178	– 482
Hypothyroid	"	355	163	– 485
Hyperthyroid b*	34th–37th	644	366	– 925
" c	"	605	243	– 754
Euthyroid b	"	577	349	– 851
" c	"	474	151	– 544
Hypothyroid b	"	474	217	– 619
" c	"	560	184	– 654

* b = burned; c = control.

* These studies were sponsored by a grant from the Armed Forces Special Weapons Project.

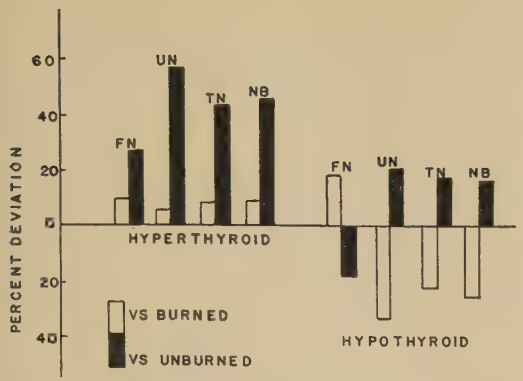

FIG. 1. Comparison of nitrogen excretion of hyper- and hypothyroid rats (burned) with euthyroid rats. FN, fecal nitrogen; UN, urine nitrogen; TN, total nitrogen; NB, nitrogen balance.

ance, 23%. Euthyroid burned rats excreted 131% more urinary nitrogen, 49% more total nitrogen, and nitrogen balance increased negatively 57%. The urinary nitrogen, total nitrogen and nitrogen balance of the burned hypothyroid rats varied +18%, —7% and —5% when compared to unburned hypothyroid controls. The deviations of hyperthyroid and hypothyroid rats from euthyroid animals is summarized in Fig. 1. The I^{131} uptake by the thyroids of the hypothyroid rats was about 3% of euthyroid (100%), whereas the uptake by the hyperthyroid animals was 6.9%. Only the burned euthyroid rats showed significant changes in I^{131} uptake (Table II). These results confirm the thyroid status of each group (12).

Discussion. The data presented here indicate thyroid status to be related to the increased nitrogen catabolism which follows severe burning. This is in agreement with the findings of others(8) who have shown no significant differences in the nitrogen excretion of burned thyroidectomized rats. Their results are similar to those obtained with the hypothyroid rats in this study. The stimulation of thyroid activity following burning(10) does appear to bear some relation to the greatly increased nitrogen excretion. The fact that burned euthyroid rats excreted nearly as much nitrogen as the burned hyperthyroid rats suggests that these groups were catabolically equal. I^{131} uptakes by the thyroids of these two groups indicate the hyperthyroid group to have taken up much less iodide than did the burned euthyroid rats. This supports the hypothesis that nitrogen catabolism in the post-burn state is related to changes in thyroid activity. Thus the insignificant changes in the nitrogen catabolism of the burned hypothyroid rats should be an indication of a less active thyroid. This is apparent in view of the low uptake of radio iodide by the thyroids of the group, which was only 6.9% of that of the burned euthyroid rats. These studies do not rule out the probability of other endocrine functions being involved in initiating or perpetuating the abnormal protein catabolism of the post-burn state.

TABLE II. I^{131} Uptake by Thyroids of Burned and Unburned Rats.

Group	cpm/avg thyroid	% of euthyroid (unburned)
A. Unburned		
Hyperthyroid	139 ± 12*	6.9
Euthyroid	2015 ± 45	100
Hypothyroid	64 ± 7	3.2
B. Burned		
Hyperthyroid	210 ± 15	10.4
Euthyroid	3569 ± 59	177.3
Hypothyroid	54 ± 7	2.7

* Stand. error.

Summary. Protein catabolism in the post-burn state appears to be closely related to thyroid status. The post-burn nitrogen excretion of euthyroid rats approached that of hyperthyroid animals, whereas nitrogen catabolism of hypothyroid rats was practically unchanged.

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Effect of Sodium 5-Allyl-5-(1 methylbutyl) 2-Thiobarbiturate on Uptake of I^{131} by Rat Thyroid. (20574)

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Recent observations(1,2) indicated sodium ethyl(1-methylbutyl) thiobarbiturate (Pentothal), a commonly used anesthetic agent, to reduce the I^{131} uptake by rat thyroid to the extent of 89%. The reduced uptake was not found to be related to the anesthetic effect, since other anesthetics not containing a $-NH-\underset{SH}{C}=N-$ grouping did not impair I^{131} incorporation into the thyroid. It was decided to test another commonly used sulfur containing anesthetic(3,4), sodium 5-allyl-5 (1 methylbutyl) 2-thiobarbiturate (Surital) to determine its effect on the I^{131} trapping mechanism and particularly the chemical partition of I^{131} in the thyroid gland.

Methods. Eight adult male rats of the Wistar strain (180-205 g) were given Surital (40 mg/kg) intraperitoneally. Sixty minutes later, each animal was given intraperitoneal injections of 2 μc of carrier-free radioiodide (I^{131}) contained in one ml of physiological saline. In addition, 8 male rats of comparable weight were given I^{131} only to serve as controls. Four hours post administration of the I^{131}, the animals were sacrificed by a sharp blow on the head, the thyroids removed, stripped of adherent tissue. Mounting and radio assay technics have been described elsewhere(2). Results are expressed as counts per minute (cpm) per milligram of tissue. Thyroids from each group were pooled immediately following radio assay and digested in 3.5 N KOH for 8 hours on the water bath. The alkaline digests were fractioned with n-butanol by the method of Tong and Chaikoff(5) with minor modifications. The radioactivity of these fractions was determined under conditions of identical geometry. Counting was continued to give a statistical error of 2% or less. Results are expressed as the percentage of total recovered radioactivity from the thyroid hydrolysate.

Results. The data in Table I indicate that Surital markedly inhibits the uptake of I^{131} by the rat thyroid. Table II shows that although the total incorporation into the inorganic, di-iodotyrosine and thyroxine fractions was much lower in the Surital treated rats than in the controls, the ratio of I^{131} among these fractions is essentially the same.

Discussion. Obviously, the inhibition of I^{131} uptake in the presence of Surital is a reaction which prevents iodide from ultimately combining with thyro-protein, (a) by in-

TABLE I. Effect of Surital on I^{131} Incorporation into Rat Thyroid.*

	cpm/mg thyroid	% controls
Control animals	107.1 ± 11.8†	100.0
Surital-treated animals	10.4 ± 1.5	9.7

* Avg of 8 animals.
† Stand. error of the mean.

TABLE II. Partition of Recovered I^{131} in Alkaline Hydrolysates of Pooled Rat Thyroid Tissue.

Thyroid fraction	Control cpm	Control % of recovered I^{131}	Treated cpm	Treated % of recovered I^{131}
Iodide	2860	56.9	320	54.1
Di-iodotyrosine	1400	27.9	196	33.1
Thyroxine	760	15.2	76	12.8
Totals	5020	100.0	592	100.0

hibiting the oxidation of I^- to I_2 or (b) by reacting with I_2 to form some other compound so making the iodine unavailable to thyroid cells(6). The same ratio of activities among the thyroid fractions from both groups of rats suggests that Surital does not interfere with thyroxine synthesis in the cells. Iodine has been shown to react with thioureas and thiouracil derivatives(7), the reaction being not always stoichiometric. This suggests that iodine may react with the thiobarbiturate forming a complex substance from which the iodine cannot be assimilated by the thyroid gland. The action of Surital probably follows the mechanism of action of other antithyroid drugs(6). This study suggests that clinical evaluation of thyroid status via I^{131} uptake should exclude previous treatment with thiobarbiturate anesthetics.

Summary. 1. Surital markedly reduces the incorporation of I^{131} into rat thyroid. 2. The proportion of recoverable I^{131} in the inorganic and organically bound fractions of thyroid alkaline hydrolysate is essentially the same for control and Surital treated rats.

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Incorporation of C^{14}-Carboxyl-Labeled Leucine by Antibody Protein.* (20575)

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An important question in the physiological behavior of proteins is whether proteins can exchange amino acids without losing their original identity. Antibody protein offers an excellent tool for this problem since such proteins can be introduced into the circulation of an animal and removed after suitable time and specifically recovered by precipitation with a homologous antigen. Heidelberger *et al.*(1), carried out an investigation of this type by feeding N^{15} labeled glycine to rabbits which had been given an injection of antipneumococcus rabbit serum. Although subsequent analysis of their passively transferred antibody precipitates gave heavy nitrogen values which were slightly higher than experimental error, they concluded *a priori* that this was the result of non-specific absorption of other serum constituents which had taken up considerable amounts of the labeled glycine. When C^{14} became available, Kooyman and Campbell(2) reinvestigated this problem by injecting C^{14} labeled leucine into rabbits which were also given injections of several different antibodies. Their results indicated that passively transferred homologous antibody acquired considerable radioactivity after several days. However, as a result of some subsequent investigations, the question arose as to whether this activity might not be the result of absorption of non-specific serum proteins as Heidelberger *et al.* had thought possible. In view of this possibility the following investigation was carried out to repeat the previous studies with leucine but to study the problem of contamination more carefully.

Materials and methods. Synthesis of C^{14}-labeled leucine. The synthesis of leucine was

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† Contribution No. 1840.

carried out by the method developed by Dr. Kooyman with only a few minor modifications. This method consisted essentially of reacting a solution of isoamyl magnesium bromide with dry carbon dioxide generated from radioactive barium carbonate.‡ The resulting isocaproic acid was brominated, treated with liquid ammonia and the leucine obtained was recrystallized as described by Marvel(3). The details of this synthesis are hardly necessary at this time since Borsook *et al.*(4) have described a superior method using the Strecker synthesis.

Determination of radioactivity. Protein samples were dried with alcohol and ether and deposited as a fine powder on counting planchets. Complete drying was necessary in order to obtain uniform surfaces on the samples. All the preparations gave similar self-absorption curves. Radioactivity determinations were made with a Geiger-Mueller counter using a Tracerlab 64 scaler. The Geiger tube had an end window thickness of 1.4 mg per cm^2. The final values were corrected to counts per minute per 10 mg of sample making the necessary correction for self-absorption.

Experimental. The first experiment was made on an 8 lb rabbit (rabbit 63) which had been receiving immunizing injections of bovine globulin p-azophenylarsonate (RBG). The last immunizing injection was given on the day that the experiment started. Intra-abdominal injections each consisting of 30 ml of a 1.0% solution of the labeled leucine were then given to this rabbit on 3 consecutive days (day 0, 1 and 2). The leucine solution was sterilized by filtration and showed an activity of about 10^5 counts per ml per minute in the counter used. On day 3 the rabbit received 2 intravenous injections of a rabbit antiovalbumin globulin solution spaced about 6 hours apart. This amounted to a total of 27 ml of solution containing 33 mg of protein per ml and approximately 6 mg of precipitable antibody per ml. On the next day (day 4) the animal received 11 ml of horse anti-SSS I and II refined serum (Lederle Labs., Inc.) which contained 49 mg of protein per ml and 12.5 mg of precipitable antibody to type I pneumococcus polysaccharide and 17.0 mg of precipitable antibody to type II pneumococcus polysaccharide per ml. On day 5, 70 ml of blood were taken from the rabbit's ear and this was defibrinated by shaking with glass beads. The rabbit then received 35 ml of sterile saline. On day 7 60 ml of blood were taken from the ear and defibrinated. The defibrinated blood samples were centrifuged and the serum was removed. Small portions of the serum samples were titrated with bovine globulin p-azophenylarsonate to determine the optimum ratio. Using this ratio the active antibody was precipitated from 20 ml portions of the sera. The samples were allowed to remain 48 hours in the refrigerator and the precipitate was then centrifuged down, washed 3 times with cold saline, and prepared for counting. Background was determined before and after each sample was counted. The radioactivities measured with the nine-tenths statistical error are given in Table I. As is shown the RBG antibody precipitates contained appreciable amounts of radioactivity.

TABLE I. Radioactivity of Antibody Proteins from Rabbit 63, Immunized against Bovine Globulin p-Azophenylarsonate and Injected with C^{14}-Labeled Leucine.

Bleed	Wt, mg	Counts/min.	Counts/min. /10 mg
	Active antibody—anti-RBG precipitate		
1	4.3	35.7 ± 2.5	81
2	10.8	58.3 ± 2.5	68
3	18.6	36.8 ± 2.0	32
	Passive homologous antibody—antiovalbumin precipitate		
1	2.7	.5 ± 1.1	
	Repeat	1.6 ± 1.8	
	2.7	.06 ± 1.0	
	Repeat	1.25 ± .8	
2	2.4	−.10 ± 1.0	
3	6.5	.03 ± .8	
	Passive horse antibody—anti-SI SII precipitate		
1	9.4	8.0 ± .4	10
2	2.4	2.0 ± .4	8

Errors given are all 9/10 statistical error.

In the earlier work the question was raised as to the effect of complement and perhaps other non-antibody protein being bound by the precipitates. The presence of radioac-

‡ Obtained from the Oak Ridge National Laboratory operated by Union Carbide and Carbon Corp.

tive material would have little effect upon the specific activity of the active antibody precipitate but could have an effect on precipitates of low specific radioactivity. Assuring the removal of a material as complex as complement would be very difficult, for although complement activity is destroyed by heating, the inactive form might still be absorbed. However, supernatants from the active antibody precipitations which should have little complement activity, if any, were heated at 56°C for half an hour. They were then tested in the standard manner for complement activity(5) and found to be negative. This still did not completely assure the removal of any material which might be carried down with the precipitates in a more or less non-specific manner but it at least made it rather unlikely. The solutions were recentrifuged and titrations were run with ovalbumin to determine the optimal ratios for precipitating the passive rabbit antibody and the precipitations and counting were carried out as for the active antibody. In a similar manner the horse antibodies were precipitated with a mixture of the two polysaccharides in the optimum proportions and counted. The results are also shown in Table I. It was found that the passive homologous antibody contained little or no activity but the horse antibody precipitates contained some activity. This of course is believed to be due to the absorption of other serum components.

These results were contrary to the earlier work of Kooyman and Campbell and it was decided to repeat the experiment with the same animal. The rabbit received a few more injections of the immunizing antigen and then on day 24 it received an intraperitoneal injection of 30 ml of the 1% tagged leucine solution. Over the next 2 days it received 4 intravenous injections of 10 ml of a rabbit antiovalbumin serum which contained about 4 mg of precipitable antibody per ml. On the next (day 27) 55 ml of blood were taken from the animal and this was treated in the same manner as were the previous samples. The results are given in Table I and again show that the transferred rabbit antibody contained no radioactivity.

For a further check on the above results the experiment was repeated with a different animal and different antigen-antibody systems. A healthy female rabbit (Rabbit 361) weighing about 7 lb received a long series of injections of crystalline bovine serum albumin (BSA) solution and had a high titer of anti-bovine serum albumin antibodies. On the 3 days following the last immunizing injection (day 0, 1 and 2) it received 27, 24 and 20 ml of a 1% solution of the labeled leucine. On the next 2 days (day 3 and 4) it received 4 10 ml intravenous injections of a solution of rabbit anti-bovine globulin p-azophenylarsonate serum. This material gave 10.9 mg of precipitate per ml when titrated with bovine globulin p-azophenylarsonate. The rabbit was then bled on days 5, 6, 13, 20 and 27 taking 40, 65, 40, 30 and 30 ml of blood on the respective days. The bleedings were all from the ear and after each bleeding the animal received an intraperitoneal injection of sterile saline. Between the third and fourth bleedings the immunizing injections with bovine serum albumin were again continued. The serum from the bleedings 1 to 5 gave 3.8, 1.8, 2.1, 1.6 and 0.0 mg of precipitate per ml when titrated with BSA and 1.8, 1.0, 0.0, 0.0, 0.0 mg of precipitate per ml when titrated with RBG. The samples were treated in the same

TABLE II. Radioactivity of Antibody Proteins from Rabbit 361, Immunized against Bovine Serum Albumin and Injected with C^{14}-Labeled Leucine.

Bleed	Wt, mg	Counts/min.	Counts/min. /10 mg
	Active antibody—anti-bovine serum albumin precipitate		
1	9.5	29.0 ± 1.4	36
2	10.8	34.5 ± 1.4	40
3	6.7	13.3 ± 1.0	21
4	1.1	1.8 ± .4	16 ± 5
5	No antibody		
	Passive antibody—anti-RBG precipitate		
1	18.0	2.8 ± 1.5 2.2 ± 1.0	2
2	9.4	3.8 ± 1.0	5
3, 4, 5	No antibody		
	Recounts		
1	10.8	1.4 ± 1.0 2.2 ± .6	2
2	6.2	.9 ± 1.2	1.5 ± 2
	Repeated with fresh material		
2	9.6	2.4 ± 1.1	3

Errors given are all 9/10 statistical error.

manner as before, checking for complement activity after the removal of the active antibody. In all cases the active antibody was precipitated first. The amount of radioactivity in each precipitate is given in Table II. It is to be noted that the passive antibody in this case did show some radioactivity although not as much as was found in the earlier work. To determine if this radioactivity was merely the result of absorption the passive antibody precipitates were resuspended in saline and again washed by the procedure outlined. The material from the second bleeding showed a real decrease in the amount of radioactivity. A different sample of the passive antibody material from the second bleeding was washed in a more thorough fashion than the other sample and it was found to contain an intermediate amount of radioactivity. The data are shown in Table II.

It seemed of interest to compare the amount of radioactivity in the antigen-antibody precipitates with that present in the other blood components that were available. The fibrin from each bleeding was washed, dried, and pulvarized and then rewashed and samples for counting were prepared in the same way as the antigen-antibody precipitates. The fibrin activities are given in Fig. 1 and 2. Samples of the erythrocytes from each bleeding were washed repeatedly with saline and were laked with distilled water. Ninety-five per cent ethyl alcohol was added to the clear solutions and the precipitates were centrifuged

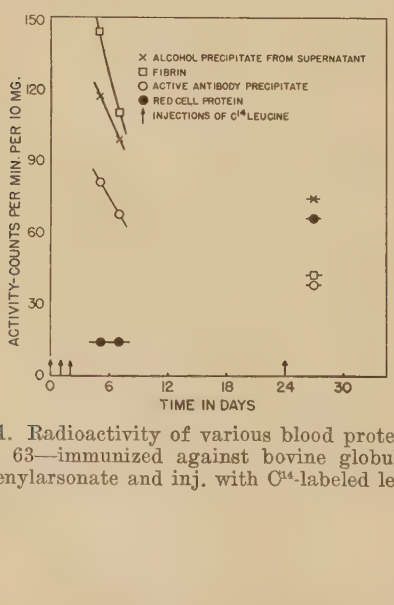

FIG. 1. Radioactivity of various blood proteins in rabbit 63—immunized against bovine globulin p-azophenylarsonate and inj. with C^{14}-labeled leucine.

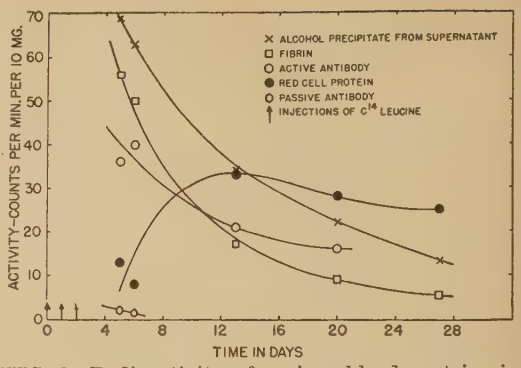

FIG. 2. Radioactivity of various blood proteins in rabbit 361—immunized against bovine albumin and inj. with C^{14}-labeled leucine.

out. The precipitates were treated in the same manner as the other materials and assayed for radioactivity. The amounts of radioactivity found are given in Fig. 1 and 2. The supernatants from the antibody precipitations were treated with alcohol and the resulting precipitates were likewise treated, mounted, and assayed for radioactivity. The amounts of radioactivity found in this material are also given in Fig. 1 and 2. The data for the antigen-antibody precipitates is also plotted in Fig. 1 and 2.

Discussion. The results obtained in these experiments on the incorporation of a C^{14} labeled amino acid by rabbit antibodies are essentially what one would predict from the heavy nitrogen experiments of Heidelberger *et al.*(1) and support their conclusion that the passive antibody does not incorporate the labeled amino acid. As shown above, in some cases the passive antibody material did contain some C^{14} but this could be removed at least to some extent by extended washing of the precipitate and would thus seem to be non-specific. It is to be noted that the passive horse antibody was even more radioactive than the passive rabbit antibody, again indicating that the effect is non-specific. In general great care must be exercised in evaluating the meaning of radioactivity in precipitates such as obtained in these experiments. A great deal of the radioactivity may be non-specific(6). This may or may not account for the earlier results. In some of the earlier experiments the passive antibody was precipitated before precipitating the active antibody and one might expect more

non-specific material to be brought down in the first of a series of antigen-antibody precipitations.

It is possible to estimate the half life of the different serum constituents from curves similar to those of Fig. 1 and 2 if it is assumed that the radioactivity is not part of a molecule that is reutilized to make more of the same material. However, as large samples of blood were taken at each bleeding the data are not too significant in this regard. It is interesting to note that the red cells are still incorporating activity when the activity of the other serum constituents is decreasing rapidly.

These results confirm those of Schoenheimer and Heidelberger. The normal constituents of a rabbit's serum incorporate radioactivity given in the form of an amino acid at a very rapid rate. Passively transferred antibodies either do not incorporate this radioactivity to any marked extent or else do so only upon the loss of their specific antibody characteristics.

Summary. C^{14}-labeled leucine has been administered to immune rabbits. It has been found that while the normal serum components incorporate the C^{14} activity passively transferred antibody does not.

The authors wish to acknowledge the advice and assistance of Professor Carl Niemann, Dr. E. Kooyman and Mr. Bror Clark.

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Studies of 11-Alpha Hydrocortisone* in the Chick Embryo and on Skin Lesions in Man.† (20576)

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Cortisone acetate has a marked, characteristic effect in modifying and inhibiting growth of the developing chick embryo(1). Because of the specificity of this effect, the chick embryo has been used to examine related steroids for "cortisone activity"(2,3), and affords a useful and simple method for testing compounds for adrenocortical activity. Compound F (17-hydroxycorticosterone) acetate was found to be 50 to 100 times more active than cortisone acetate in the chick embryo and the newly-hatched chick(4); this marked difference was not noted in comparative studies on mammals.

The growth-inhibiting dose of hydrocorti-

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TABLE I. Effects of Hydrocortisone Acetate and 11-alpha (Hydroxy Epimer) of Hydrocortisone ("Epimer") when Injected onto Chorioallantoic Membrane of the 8-Day-Old Chick Embryo.

Dose/egg, mg	No. eggs	No. surviving, sacrificed at 18 days	Avg wt, g	Avg steroid effect "Stage"
Controls	20	17	17.4 ± 2	0
"Epimer"				
.5-1	6	5	17.8 ± .8	0
2	11	11	18.7 ± 1.7	0
5	27	13	15.4 ± 2.8	0
Hydrocortisone acetate				
.005-.01	13	11	16.7 ± 3.24	0
.02	28	21	10.0 ± 2.4	1.5 ± 1
Combination Epimer, 5 Hydrocortisone acetate, .02	20	10	9.0 ± 2.2	2 ± 1

sone acetate is in the range of 0.01 to 0.02 mg/egg; free hydrocortisone is about 1/5 as active (0.1 mg/egg). The high activity of hydrocortisone in the chick embryo made this organism particularly useful for studying the effects of analogues of hydrocortisone. This report describes the inactivity of the 11-alpha hydroxy epimer of hydrocortisone in the chick embryo; this was also confirmed by another procedure, whereby the 11-alpha epimer, hydrocortisone and cortisone were injected into human skin lesions in which the latter steroids previously had been found to induce regressions(5).

Materials and methods. White Leghorn embryos were used and incubated at 38°C at a relative humidity of 85%. The test steroid was suspended in saline and inoculated on the chorioallantoic membrane (CAM) of the 8-day embryo. In some cases, where it was necessary to test large amounts of steroid, the substance was applied directly as a powder onto the CAM. The CAM was examined when the embryo died or was sacrificed and, in most cases, it was grossly clear, indicating the steroid had been absorbed. The eggs were candled daily and the dead embryos examined grossly. Embryos surviving to 18 days were weighed, and the degree of steroid effect graded according to the method previously described(1). This grading system extends from Stage I (slight effect), Stage II (definite effect), to Stages III and IV (the maximum degree of adrenocortical steroid effect). Several experiments were done with each compound. The results obtained were pooled and analyzed together. While the trend of the experiments was similar, the fact that several experiments were pooled explains in part the wide standard deviations in the data.

For the clinical study, two patients were selected with multiple skin lesions, measuring 5 to 15 mm. One was a 27-year-old colored female with disseminated Boeck's sarcoid of 5 years duration; the other a 72-year-old white male with generalized Hodgkin's disease. The nature of the lesion was established by microscopic examination of a skin biopsy. Selected skin lesions were infiltrated with 0.1 cc of a suspension of the following: a) 5 mg (50 mg/cc sol.) and 2.5 mg (25 mg/cc) of hydrocortisone acetate. b) 5 mg (50 mg/cc) and 2.5 mg (25 mg/cc) of cortisone acetate. c) 5 mg (50 mg/cc) and 2.5 mg (25 mg/cc) of the 11-alpha epimer of hydrocortisone. d) 0.1 cc of the cortisone and hydrocortisone suspending agent, and 0.1 cc of a 1% solution of carboxymethylcellulose used to suspend the 11-alpha hydroxy epimer of hydrocortisone.

Results. 1. Lack of adrenocortical activity of 11-alpha hydroxy epimer of hydrocortisone in the chick embryo. The "epimer" was applied to the CAM in a solution, in most cases, but as a powder at the high doses. Table I shows that doses of "epimer" ranging from 0.5 to 5 mg per egg were without demonstrable growth-inhibiting or adrenocortical activity in the chick embryo. At 5 mg there was a

slight suggestion of growth-inhibition. Embryos did not tolerate larger doses of the "epimer" in most cases. Three embryos, however, that survived 10 mg doses of the "epimer" showed slight growth-inhibition and questionable evidence of an adreno-steroid effect. It is concluded that the 11-alpha hydroxy epimer of hydrocortisone at tolerated doses is without appreciable adrenocortical-like activity in the chick embryo.

2. Lack of effect of the "epimer" on skin lesions in man. The skin lesions infiltrated with steroids gave the following results: a) hydrocortisone acetate caused complete or nearly complete regression within 7 days. b) cortisone acetate produced a significant reduction of 50 to 70% in size within 7 to 10 days, but the response was somewhat less than with hydrocortisone. c) the "epimer" and the suspending agents produced no measurable change. The untreated lesions were unchanged.

3. Failure of 11-alpha hydroxy epimer of hydrocortisone to modify the action of hydrocortisone acetate in the chick embryo. In Table I it is observed that 0.02 mg Compound F acetate produced a consistent modification in the growth of the chick embryo. In a group of embryos, hydrocortisone acetate, 0.02 mg per egg, was given together with 5 mg of the "epimer". When the surviving embryos were sacrificed at 18 days there was no evidence of any antagonistic effect of the "epimer" in interfering with the activity of hydrocortisone acetate. If anything, the embryos receiving the combined treatment were slightly more affected, suggesting either that the 2 agents may have had an additive effect on the chick embryo, or that the "epimer" contained a small percentage of hydrocortisone as an impurity. This difference, however, was not statistically significant, and it was concluded that the 11-alpha hydroxy epimer does not significantly antagonize or synergize the effect of hydrocortisone acetate in the chick embryo.

Discussion. Our experience with 11-α-hydroxycorticosterone indicates that it was without appreciable adrenocortical activity in the chick embryo, even when given at 200 to 300 times the effective dose of hydrocortisone acetate and 40 to 50 times that of free hydrocortisone. This loss of biological activity is presumably due to the fact that the hydroxy group in the 11 position has been changed from the natural beta orientation to the unnatural alpha configuration. It is of interest that this change in the orientation of the 11-hydroxyl group appears to inactivate the molecule almost completely. On the other hand, 11-desoxy hydrocortisone acetate (Compound S), that is, hydrocortisone without an 11-hydroxy group, has a definite inhibitory effect on chick embryo development; the minimum effective dose necessary to produce stunting of the embryo is 2.7 mg/egg as compared to 0.01 to 0.02 for hydrocortisone acetate(3). It is thus evident that the 11-alpha hydroxy group not only inactivates hydrocortisone but is actually a hindrance to biological activity, since the 11-alpha hydroxy epimer is even less effective than the analogously constituted 11-desoxy steroid (Compound S).

Summary and conclusions. 1. The 11-alpha hydroxy epimer of hydrocortisone does not inhibit the growth of the chick embryo when given at doses up to 5 mg/egg; this is 200 to 300 times the dose level at which hydrocortisone acetate (0.01 to 0.02 mg/egg) and 50 times the level at which free hydrocortisone (0.1 mg/egg) interfere with embryonic development. The unnatural orientation of the 11-alpha hydroxy group thus inactivates the molecule as far as inhibiting embryonic growth is concerned. This unnatural configuration actually inhibits biological activity, since an analogous compound, 11-desoxy corticosterone acetate (Compound S) inhibits embryonic growth at a dose level of 2.7 mg/egg. The epimer does not diminish or potentiate the action of hydrocortisone acetate in the chick embryo.‖ 2. The 11-alpha hydroxy epimer of hydrocortisone in

‖ After this work was submitted for publication, a report by Segaloff, A., and Horwitt, B. N., *Science*, 1953, v118, 220 appeared, describing the limited activity of "epi-F" in causing liver glycogen deposition in adrenalectomized mice, and its inability to interfere with the glycogen deposition produced by hydrocortisone. These results are in agreement with our own.

contrast to hydrocortisone acetate and cortisone acetate is also inactive by intra-tumoral injection in producing involution of the skin lesions in patients with Boeck's sarcoid and Hodgkin's disease.

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Excretion of Ether-Soluble Acids by Rats on Necrogenic Diet with and Without Supplements of Antibiotics.* (20577)

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Antibiotics such as aureomycin and penicillin when added to a necrogenic diet prolong the life of rats by delaying the onset of liver necrosis(1). Regardless of whether the antibiotics act by systemic action on the animal or by interaction with the intestinal flora, indirect effects should appear in the metabolism of the host. For this reason a comparison of the excretion products of rats on a necrogenic diet with those of animals receiving supplements of aureomycin or penicillin is of interest. The present study deals with the effect of the addition of these antibiotics to the basal necrogenic diet on the ether-soluble acid excretion of the rats.

Animals and methods. Male weanling rats (Sprague-Dawley or Carworth Farms) initially weighing approximately 45 g were kept in individual cages and were fed the necrogenic basal diet† with a daily supplement of vitamins(1). Aureomycin-HCl (Lederle) 25 mg, penicillin (N N′-Dibenzylethylene diamine-Wyeth) 5 mg, or vit. E 1 mg, were added to the daily rations of the treated animals. Control animals were offered 8.0 g of diet daily and the treated animals were pair-fed. For the *collection of urine* rats were placed in metabolic cages and kept on regular rations with water *ad lib.* Urine was collected under toluene. Funnels were washed down with hot water every day and the urine filtered and frozen. Analyses were run in duplicate on aliquots of combined 2- or 3-day samples of non-hydrolysed urine. *Nitrogen* was run by a semi-micro Kjeldahl method(2). Creatinine was run by the Jaffe reaction(3). Ammonia and urea after hydrolysis were determined by aeration and titration(4). Para aminobenzoic acid was determined by the usual diazotization method(5). Ether-soluble acids in urine were extracted by the method of Kanzaki(6) as modified by Bray *et al.*(7). Urine samples (10 ml) acidified with sulfuric acid (1 cc, 1 N) were continuously extracted with ether for 3 hours, the ether evaporated, the residue taken up in water and titrated with 0.1 N NaOH solution. Duplicate extracts of the same urine gave closely similar titration values. Hippuric acid (20 mg) added to a sample of urine was recovered 95-100% under the conditions of the experiments. Hippuric acid was determined after destruction of urea of the ether-soluble extract(8). Chromatograms of ether-soluble acids were run by descending technic with a butanol-acetic acid-water mixture (4:1:1) as solvent(9). Spots were developed with an alcoholic solution of brom cresol green.

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† Basal diet consists of: Corn starch 79; Baker's yeast 18 (British "D.C.L. Vit. B_1 yeast" from Distillers Corp., Ltd.); Salt Mix No. 2 USP 3; Peanut oil 0.5.

TABLE I. Effect of Aureomycin and of Penicillin on Daily Excretion of Ether-Soluble Acids in Rats on Necrogenic Diet.

Supplement to basal necrogenic diet	No. of days rats were on diet: 1–40 No. rats tested	1–40 ESA, μM × 10 / Creatinine, μM*	1–40 Diff. from control	40–200 No. rats tested	40–200 ESA, μM × 10 / Creatinine, μM	40–200 Diff. from control	Statistical significance (P) of diff.
0 (control)	5	88 ± 2.8		10	142 ± 12.4		—
Vit. E	8	100 ± 3.2	12	8	137 ± 7.5	5	0
Aureomycin	7	65 ± 10.4	23	14	77 ± 3.6	65	P 0.01
Penicillin	6	103 ± 4.0	15	12	67 ± 4.5	75	P 0.01

* ESA/N gives similar results: Daily quantities of creatinine and nitrogen were constant in each rat during duration of exp. There was no difference between the amounts excreted daily by rats from different groups.

Two of the ether-soluble acids found were crystallized. Two hundred ml of urine was extracted with ether for 20 hours. The ether fraction was then extracted with 200 ml 0.1 N $NaHCO_3$. The $NaHCO_3$ solution was acidified with H_2SO_4 to pH 1-2, decolorized with Norit A, and filtered. The filtrate was extracted with 200 ml ether, and the extract was evaporated. The residue was taken up in a small amount of ether, and the acid was precipitated out of solution by dropwise addition of petroleum ether in the cold. The two acids identified were crystallized directly from two different specimens of urine; no separation of the two acids was necessary.

Results. Urines of rats on a necrogenic diet were compared with that of treated animals of comparable weight, age, and food intake. The excretion of para-aminobenzoic acid, ammonia, urea, hippuric acid, nitrogen, and creatinine was similar in all the rats.

No differences in the quantity of ether soluble acids in the urine of treated and non-treated rats was observed during the first 40 days on the diet. Most rats kept on the necrogenic diet succumb to liver necrosis after 30 to 60 days, but a few survive. In these rats a statistically significantly higher excretion of ether soluble acids was found than in comparable animals protected with antibiotics (Table I). The animals given vit. E also showed an excretion rate comparable to the animals on the necrogenic diet. The pattern of spots in chromatograms of the ether soluble acids excreted by rats treated with antibiotics is different from that of non-supplemented animals or from animals given supplements of vit. E. These differences are shown most clearly in the chromatograms of the ether soluble acids of the older rats (Fig. 1). Ether soluble acids from the urine of non-treated or vit. E-treated rats showed spots at R_f 0.86 and 0.95. A few of these rats also showed spots at R_f 0.45. The rate at which these rats died was apparently unrelated to the presence or absence of the acid of R_f 0.45.

In contrast, almost all the rats treated with antibiotics have acids of R_f 0.45, R_f 0.86 and 0.95. In addition, all the rats treated with antibiotics have an additional acid of R_f 0.80. Hippuric acid gives a spot of R_f 0.93 and

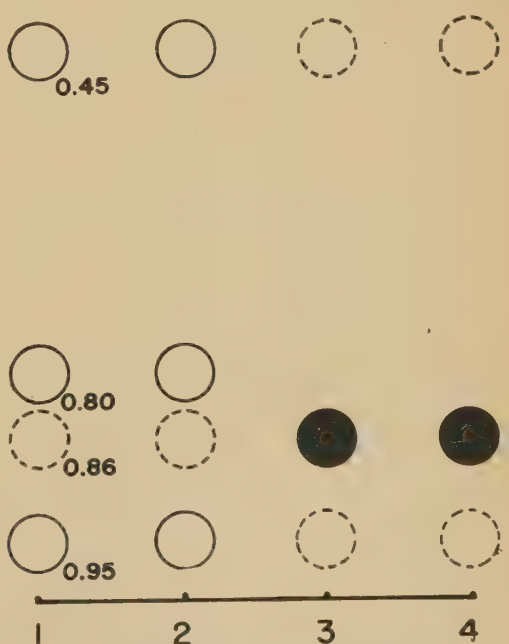

FIG. 1. Typical descending chromatogram of ether soluble acid excretion by rats on necrogenic diet with supplements 1. penicillin; 2. aureomycin; 3. necrogenic; 4. vitamin E.

PABA 0.92 under the conditions of our experiment. Five micromoles of total acid were found to give the clearest separation of spots; and this quantity was also used for test substances.

The acid of R_f 0.86 was crystallized from the urine of rats given vit. E by the method described above. The crystals were found to have a melting point of 129, an equivalent weight of 65, and elemental analysis C:46, H:8, O:64 (by difference), and were identified as methyl malonic acid. Mixed melting point, solubility in water, alcohol, ether, and petroleum ether, and R_f value were identical with that of pure methyl malonic acid.

The acid of R_f 0.95 was crystallized from the urine of rats given penicillin by the method described above. The crystals were found to have: m.p. 140, equiv. wt 75, and were identified as α,α-dimethyl succinic acid. Mixed melting point, solubility in water, alcohol, ether, and petroleum ether, and R_f value were identical with that of pure α,α-dimethyl succinic acid.

The ether-soluble acids of R_f 0.45 and 0.80 have not yet been identified.

Titration of the chromatographic spots (Fig. 1) after cutting and eluting showed the following distribution:

R_f	No supplement, %	Vit. E, %	Aureomycin, %	Penicillin, %
.45	*	*	10	10
.80	None	None	25–30	25–30
.86	85–90	85–90	15–40	15–40
.95	10–15	10–15	30–60	20–50

* Too small to measure.

Discussion. Aureomycin, penicillin and vit. E, when added to the necrogenic experimental diet, caused no quantitative alteration in the ammonia, urea, nitrogen, creatinine, hippuric acid, or para-aminobenzoic acid excretion in the urine.

The significance of the ether-soluble acid excretion in the urine of rats is unknown. Presumably, these acids represent end products of the metabolism of the animals.

Both the surviving non-supplemented rats and those receiving vit. E excrete high levels of ether-soluble acids. Either the surviving animal on the necrogenic diet has undetermined vit. E reserves or has developed a pathway similar to that of vit. E-treated animals.

No high levels of ether-soluble acids were found in the urines of rats receiving antibiotics. Since antibiotics prolong the lives of the rats but do not offer the permanent protection given by vit. E supplements, a different metabolic pathway is suggested for the acid excretion of the 2 groups of rats.

Methyl malonic acid forms the largest part of the total ether-soluble acid in the untreated or vit. E-treated animal, whereas α,α-dimethyl succinic acid and acid of R_f 0.80 forms the largest part of the acid excretion of the animals treated with antibiotics. No significant quantities of homogentisic acid were found in any of these urines(10). Methyl malonic acid has been described in the urines of rats fed anthracene(11), but was apparently unrelated to death in these animals. α,α-dimethyl succinic acid has not previously been described in the urine of rats.

Summary. 1. Addition of aureomycin or penicillin to the necrogenic diet showed no effect on the quantity of nitrogen, urea, ammonia, creatinine, hippuric acid or para-aminobenzoic acid excreted in the urine by rats. 2. Most rats on necrogenic diet die of liver necrosis between 30 and 60 days; a few survive. In these rats a statistically significantly higher urinary excretion of ether soluble acids was found than in rats treated with aureomycin or penicillin. Chromatograms of these ether-soluble acids indicated that addition of aureomycin or penicillin to the diet but not vitamin E altered the components of these acids in the urine. 3. The ether-soluble acids in the non-hydrolysed urine consist of para-aminobenzoic acid, hippuric acid, methyl malonic acid, α,α-dimethyl succinic acid and at least 2 unidentified acids. Methyl malonic acid forms a large part of the total ether-soluble acids in the non-treated and vit. E-treated animal, and forms a smaller fraction of the acid excretion of the animals receiving penicillin or aureomycin.

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Leptospiral Antigen Demonstrated by the Fluorescent Antibody Technic in Human Muscle Lesions of *Leptospirosis icterohemorrhagiae.** (20578)

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Leptospiral antigen was demonstrated with the fluorescent antibody technic in the muscle lesions of a patient with *L. icterohemorrhagiae* infection.

Materials and methods. A 1 cm square piece of the left gastrocnemius muscle was excised from a 42-year-old Negro construction worker on the 9th day of an acute febrile illness, associated with icterus, azotemia, muscle pain which was particularly marked in the calf, headache and malaise. Half the specimen was immediately quick frozen in dry ice and alcohol and stored at —20°C. The remaining tissue was divided and fixed in Zenker's fluid with 5% glacial acetic acid and in 10% formalin. Routine histologic preparations revealed the typical lesions of acute Weil's disease (Fig. 1). These lesions consisted of focal areas of necrosis of muscle fibers with slight proliferation of sarcolemmal nuclei and minimal inflammatory cell infiltration(1). Sections stained by the Whartin-Starry method failed to reveal any organisms. Agglutination tests were positive for *L. icterohemorrhagiae* with titres of 1:6400 on the 16th day of illness and 1:12800 on the 31st day. Both serum samples were also positive for *L. mitis* at a titre of 1:640 and for *L. canicola* at a titre of 1:200. During convalescence, 31 days after the onset of the illness, a biopsy of the right gastrocnemius muscle was taken. This tissue was prepared as described above. Routine histologic preparations of this specimen revealed advanced healing by regeneration. Silver stains for leptospira again showed no organisms. Frozen sections of unfixed frozen muscle were prepared following the technic of Linderstrøm-Lang as modified by Coons *et al.*(2). Preliminary to staining, the mounted sections were fixed either by dipping briefly in 70% ethanol and rinsing with buffered saline or by covering with acetone for 15 minutes with subsequent evaporation of the acetone in an incubator at 37°C. These 2 types of fixation had been arrived at while adapting the fluorescent antibody technic to the study of experimental leptospiroses in Syrian hamsters.

The sections were stained for 30 minutes with the specific conjugate prepared according to the method of Coons and his associates(3). Conjugates with fluorescein isocyanate were prepared from immune sera obtained from rabbits injected with either *L. icterohemorrhagiae* or *L. canicola.*† The agglutination

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† The fluorescein amine was prepared by Dr. A. H. Coons to whom I am most grateful for his kind advice and help.

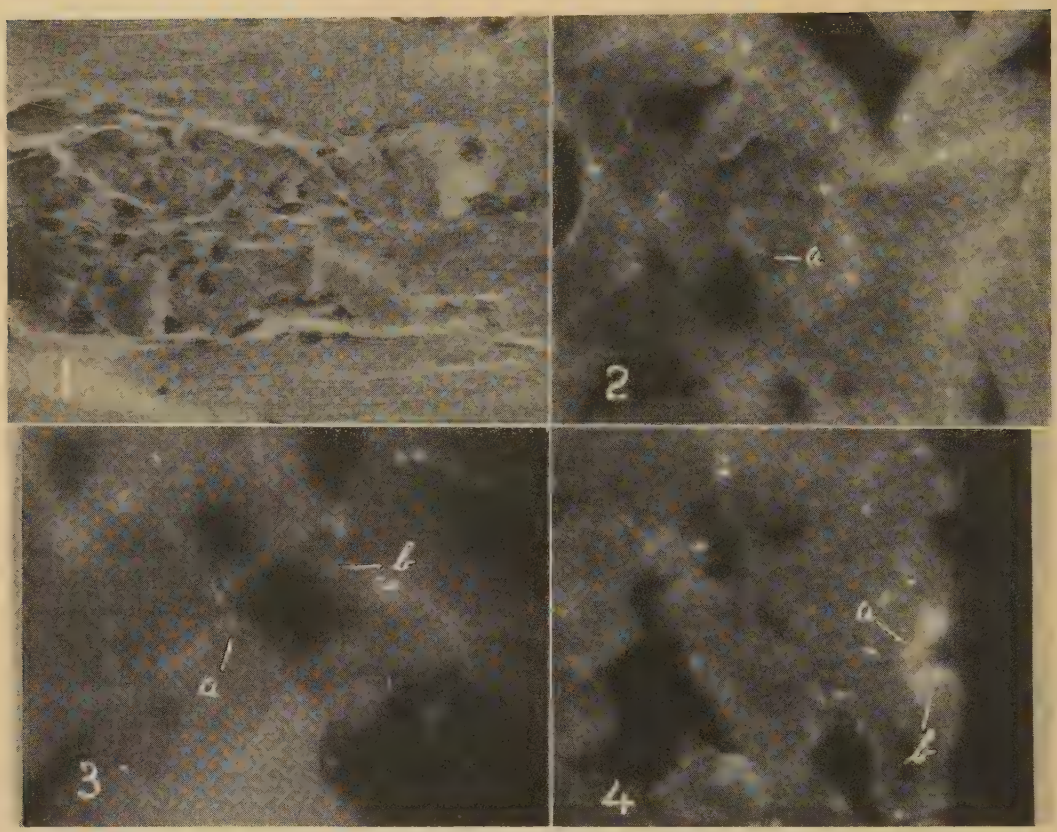

FIG. 1. Areas of focal necrosis in 2 adjacent striated muscle fibers. Hematoxylin-Phloxine. × 370.

FIG. 2. Fluorescence photomicrograph showing minute specifically fluorescing granular deposits (a) in sarcoplasm. × 370.

FIG. 3. Fluorescence photomicrograph showing 2 specifically fluorescing deposits in muscle. The larger deposit (a) is granular, the other (b) shows a delicate and slightly curved structure resembling a leptospira. × 370.

FIG. 4. Fluorescence photomicrograph showing area of muscle necrosis (a). A minute deposit of specifically fluorescing material is visible at the edge of the necrotic area (b). × 370.

titre of each serum was 1:6400. Before use for staining, the conjugate was absorbed twice with human liver powder. This procedure, however, did not eliminate the non-specific staining of human neutrophilic polymorphonuclear leucocytes, which was observed in blood smears of this patient and normal humans. It was found that the non-specific staining of these cells, which was observed also in other mammalian species could be eliminated in human as well as in rabbit tissue by absorbing the conjugate twice with acetone-dried rabbit bone marrow powder. Controls consisted of: 1. inhibition tests in which paired frozen sections were covered with unconjugated normal or specific immune serum preliminary to the staining with the specific conjugate, and 2. staining with *L. canicola* conjugated antiserum.

Results. Under the fluorescence microscope a few widely scattered deposits of specifically fluorescing green material were found. The deposits were located in a vacuolated appearing area within the sarcoplasm (Fig. 2). Only in a few instances were they encountered at the periphery of areas of muscle necrosis. They were occasionally seen at the periphery of a muscle fiber, but it could not be determined if they were located in the sarcoplasm, within the sarcolemma sheath or within the perimysium. The deposits appeared as minute granules but in rare in-

stances single elongated and curved structures resembling intact leptospirae were seen (Fig. 3). The foci of muscle necrosis were clearly visible and in general showed yellow fluorescence against the grayish-blue background (Fig. 4). Occasionally the yellow color showed a greenish fluorescing tinge. No antigen was found in the interstitial connective tissue or in the nuclei.

Fluorescent deposits were not found in sections stained with *L. canicola* conjugate or subjected to the inhibition test by treatment with unconjugated specific immune serum before staining with the specific conjugate.

Leptospiral antigen was not seen in the biopsy taken on the 31st day of illness.

Comments. The specificity of muscle lesions in Weil's disease has been questioned by Adams, Denny-Brown, and Pearson(4) since even in severe infections with florid lesions it is extremely difficult with the conventional histologic methods to identify with certainty leptospirae in the muscle lesions(5). I have studied the muscle lesions obtained by biopsy or at autopsy of many patients with *L. icterohemorrhagiae* infection. Only once have I encountered in silver impregnated sections from muscle biopsy structures which I felt certain were leptospirae. These structures were few in number and were found at the periphery of the muscle lesions. They were not present in the necrotic debris. The presence of leptospiral antigen in the muscle and its lesions seems to indicate that the lesions are produced by the organism.

The amount of demonstrable antigen in this patient was sparse. It was generally seen in the sarcoplasm not associated with well developed lesions and was found only rarely at the periphery of the foci of muscle necrosis. Antigen was not identified in the nuclei, interstitial tissues or among the debris of the lesions, although the necrotic material sometimes showed a faint greenish-yellow fluorescence. It is possible that the paucity of demonstrable antigen is due to its saturation or destruction by endogenous specific antibody since the biopsy was taken on the 9th day of illness, at which time there is a significant antibody titre in Weil's disease. The presence of a green fluorescing tint in some of the otherwise yellow fluorescing areas of necrosis suggests the diffusion of antigen among the necrotic material.

The non-specific staining of polymorphonuclear leucocytes of normal humans and other mammalian species is of interest. This staining appeared even in blood smears fixed with either alcohol or formalin and after treatment with fluoride. The suppression of the non-specific staining of these cells by absorption of the conjugate with bone marrow powder seems useful when the fluorescent antibody technic is applied to lesions associated with inflammatory cell infiltration.

Summary. Leptospiral antigen was demonstrated with the fluorescent antibody technic in the muscle lesions of a patient with *L. icterohemorrhagiae* infection. This observation indicates that the muscle lesions in Weil's disease are produced by the leptospirae. It has also been shown that the non-specific staining of human polymorphonuclear leucocytes can be eliminated by absorption of the conjugate with rabbit bone marrow powder.

The technical assistance of Miss Hillma Gheesling and Miss Elaine Schubert is gratefully acknowledged.

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Role of Serum Albumin in Lipemia Clearing Reaction. (20579)

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Earlier publications from this Laboratory (1,2) have described investigations into the mechanism of the "lipemia clearing reaction" wherein a clearing factor, produced *in vivo* by the intravenous administration of heparin, clears elementary lipemia or artificial oil emulsions *in vitro* in the presence of normal plasma. The cofactor supplied by the normal plasma was termed "coprotein". The preparation of a partially purified, presumably "coprotein"-free, clearing factor which could be used to assay for "coprotein" activity has been reported(2). Attempts were therefore made to purify "coprotein" by the alcohol fractionation methods of Cohn, *et al.*(3), as well as by classical ammonium sulfate procedures, in which it became increasingly evident that the albumin-rich fractions contained considerable "coprotein" activity. Since it was impossible to separate this activity from the serum albumin, experiments were undertaken to clarify the effect of purified albumin itself on the clearing reaction.

Methods. In the first experiments to be described, the source of clearing factor was fresh whole plasma from a dog that was anesthetized with nembutal, treated with 2.5 mg/kg heparin intravenously, and bled after 5 minutes. The purified clearing factor was prepared by the method referred to above(2). Two sources of albumin were employed: commercially available bovine Fraction V (Armour Laboratories), and a human albumin twice crystallized with mercury by the method of Hughes(4). No difference was observed in the behavior of the 2 albumin preparations in these experiments. A coconut oil emulsion supplied by Dr. Douglas Frost of Abbott Laboratories, diluted to 0.5% fat content, was used as substrate. The buffer diluent described previously(2) was employed, and all reaction mixtures contained sufficient quantities of the disodium salt of ethylenediaminetetraacetic acid to prevent the formation of insoluble calcium soaps. Reactions were run at 37°C in a waterbath, and their progress followed by optical density readings in the Coleman Junior Spectrophotometer at a wave length of 500 mμ.

TABLE I. Effect of Added Albumin in the Clearing System.

Clearing system	Decrease in optical density after 10 min.	60 min.
Heparinized D.p* alone	.012	.027
D.p + 0.1 cc 5% a*	.020	.088
D.p + 0.2 cc 5% a	.021	.088

* D.p = dog plasma; a = albumin.

Observations. Effect of added albumin in the clearing system. In a 1 cc test system containing 0.07 cc of clearing factor-containing dog plasma and 0.1 cc of coconut oil substrate, the addition of 0.1 cc of a 5% solution of albumin produced a moderate increase in the initial rate of clearing, and a marked increase in the extent of the reaction after one hour. Further additions of albumin produced no further effect within the one-hour period of observation. Table I illustrates this effect. In another type of experiment, 0.1 cc of dog plasma was allowed to act on 0.1 cc of coconut oil in a total volume of 1 cc for 135 minutes, until the rate of clearing was markedly reduced. At this time, 0.1 cc of 5% albumin was added, resulting in a greater than 3-fold increase in the rate of clearing.

Effect of fatty acid in the clearing system. Sodium oleate was added in varying amounts to a 1 cc clearing system containing 0.1 cc dog plasma, 0.2 cc 5% albumin, and 0.1 cc coconut oil substrate. Increasing inhibition of the clearing reaction was observed with increasing amounts of oleate. In Fig. 1 is shown the extent of clearing after one hour as a function of the amount of oleate added (expressed as moles of oleate per mole of albumin present, assuming that the dog plasma also contains approximately 5% albumin.) It is evident

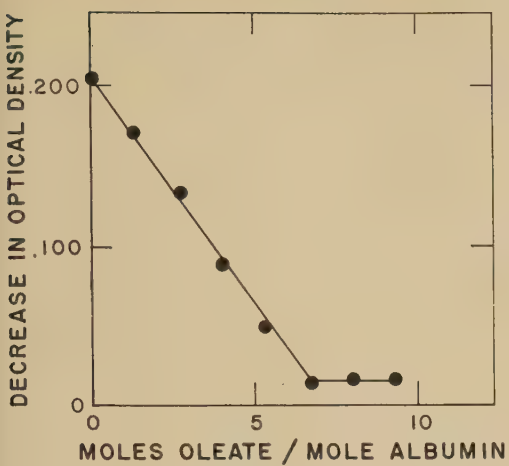

FIG. 1. Inhibition of lipemia clearing reaction by oleate.

that maximum inhibition, the reaction being virtually completely stopped, is reached when 6 to 7 moles of oleate have been added for each mole of albumin present. Sodium palmitate was found to produce inhibition in similar fashion, while glycerol in concentrations up to M/300 was without noticeable effect.

Effect of albumin and serum on purified clearing factor. In a system containing purified clearing factor and an optimal quantity of albumin, the addition of a small amount of normal serum or plasma will greatly accelerate the clearing reaction. Table II outlines a typical experiment to illustrate this effect. Each tube contained 0.1 cc of a 2% solution of purified clearing factor, 0.1 cc of coconut oil substrate, and enough buffer to make a total of 1.0 cc. It is evident that the greatest clearing was obtained in the tube containing normal serum.

Discussion. An impressive body of evidence has been accumulated showing that serum albumin, at physiologic pH values, has a considerable affinity for the anions of various organic acids, including the fatty acids(5-7). Unpublished data of Dr. H. A. Saroff indicate that at pH 4.3, each mole of albumin will bind approximately 2 moles of palmitate ion, while at pH 6.5 it can bind 6 moles per mole. Davis and Dubos(6) find a binding capacity for oleate of about 9 moles per mole of albumin (pH not stated). Cohn, Hughes, and Weare (8) indicate that, as it is prepared from plasma, albumin contains up to one mole of fatty acid per mole of protein. In our inhibition experiments, therefore, a total of 7 to 8 moles of fatty acid per mole of albumin was probably present at the point of complete inhibition of the clearing reaction.

TABLE II. Effect of Albumin and Serum on Purified Clearing Factor.

Cofactor supplied to purified clearing factor	Decrease in optical density after 1 hr	2 hr
0	.004	.009
.2 cc 5% albumin	.046	.069
.2 cc 5% albumin + .005 cc normal serum	.109	.159

It has been found(2,9,10) that during the clearing reaction, fats are hydrolyzed to liberate fatty acids. That fatty acids can inhibit the clearing reaction is shown by these experiments. It seems highly likely, therefore, that serum albumin enters the reaction by binding and thereby removing the fatty acids evolved.

The authors are not aware of published experiments concerned with the possible role of albumin in other serum-catalyzed lipolytic reactions. The highly purified preparations of serum esterase reported by Surgenor and coworkers(11,12) did not appear to lose activity with the progressive removal of albumin, suggesting that this enzyme, at least, can function in the absence of albumin.

Whole normal serum, in minute amounts, considerably enhances the effect of albumin in a purified clearing system, indicating that it provides a second cofactor which is of importance in the clearing reaction. Our present data are not adequate to decide whether this factor is an essential reactant or catalyst, or merely an accelerator. With better purification of the clearing factor it should be possible to resolve this problem. It seems logical meanwhile to reserve the term "coprotein" for this second, as yet unidentified, material in serum. Preliminary experiments indicate that it is thermolabile and non-dialyzable, hence probably protein in nature.

The discovery that two cofactors, albumin and coprotein, play simultaneous roles in the clearing reaction casts doubt on the reliability of our earlier studies on the fractionation and properties of "coprotein" (1,2). Further ex-

periments to isolate and characterize coprotein are in progress.

Summary. Data are presented to show that purified serum albumin enhances the lipemia clearing reaction, probably by binding the fatty acids produced which, if not removed, are capable of inhibiting the reaction. Evidence that albumin is not the only cofactor in the clearing reaction is presented.

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Biosynthesis of C^{14}-Labeled Benzylpenicillin. (20580)

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Although phenylacetic acid is one of the well-established precursors of benzylpenicillin, only limited attention has been given to the metabolic changes which occur when the acid is utilized by a penicillin-producing fungus. For this reason we investigated the problem in more detail. The incorporation of phenylacetic acid-l-C^{14} into benzylpenicillin has been achieved and is reported together with observations which indicate other possible pathways of phenylacetic acid metabolism.

Experimental and results. Phenylacetic acid-l-C^{14} was synthesized by the method of Dauben *et al.**(1). Two separate preparations had specific activities of 15.29×10^5 and 30.15×10^9 c.p.m. per mole.† The acid of lower activity was used in this investigation. One hundred and sixty ml of semisynthetic medium‡ in 500 ml baffled Erlenmeyer flasks were inoculated with the young (24-36 hour) vegetative mycelium of *Penicillium chrysogenum,* BC-65, Upjohn, and incubated on a rotary shaker at 25-27°C. After incubating for 24 hours, potassium phenylacetate-l-C^{14}, equivalent to 375 μg of the free acid/ml was added. At the end of the fermentation period (96 hours) the pH had risen from 5.5 to 8.4. The amount of penicillin produced was 867 μg/ml, while the control with no phenylacetic acid contained 311 μg of penicillin/ml. The harvested dry mycelium from the fermentation with the added precursor amounted to 11.2 mg/ml. A 200 ml portion of the fermented beer was acidified (pH 2.2) with 25% H_2SO_4 and extracted with amyl acetate. The combined amyl acetate extracts in turn were extracted with saturated phosphate buffer (pH 6.8). The buffer was acidified (pH 2.2) and extracted with ether. After the

* The $BaC^{14}O_3$ used was purchased from the Oak Ridge National Laboratory, Oak Ridge, Tenn.

† Measurements of radioactivity were made on the solid neutralized samples with a thin-window counting tube and autoscaler manufactured by Tracerlab, Inc., Boston, Mass. The values reported are corrected for infinitely thin samples.

‡ The medium consisted of: lactose 50 g, glucose 5 g, corn steep liquor solids 17 g, $CaCO_3$ 3 g, $FeSO_4 \cdot 7H_2O$ 0.004 g, Na_2SO_4 0.5 g; H_2O to 1000 ml; pH 5.5.

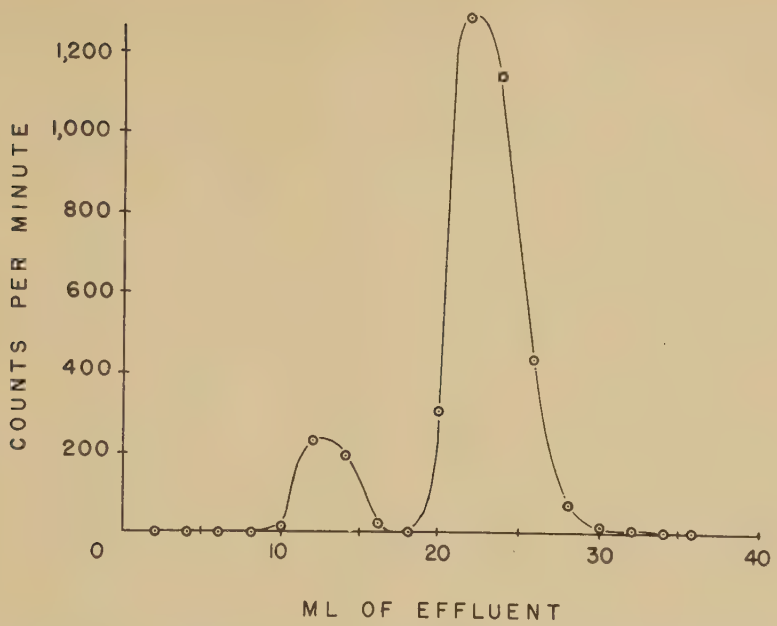

FIG. 1. Distribution of radioactivity in chromatographed fractions of crude penicillin. 1.7 mg of penicillin, 'Hyflo Supercel' column; values corrected for background.

ether was evaporated with a mild stream of air at room temperature, the resulting amorphous material was dried *in vacuo*. A portion was then dissolved in 0.2% phosphate buffer (pH 6.8) and separated by a modification of the paper chromatographic procedure of Karnovsky and Johnson(2), using water-saturated ether as the mobile phase. After development the paper was cut lengthwise into 2 strips, each 5 mm wide, in such a way that each strip contained an equal quantity of the separated material. One of these strips was used to identify the individual penicillins by bioassay on an agar layer seeded with *Micrococcus pyogenes* var. *aureus*. The other strip was used for radioautograms.

The bioassay indicated that the isolated material consisted of 94.5% benzyl-, 4.3% heptyl-, 0.9% n-amyl-, 0.3% 2-pentenyl-, and traces of p-hydroxybenzylpenicillin. The radioautograms showed that the major portion of the radioactivity was located in the area coinciding with the benzylpenicillin spot as identified by bioassay. A second diffuse spot on the radioautogram was observed in the approximate area of phenylacetic acid, the identity of which was verified in a butanol-acetic acid-water (74:19:50 ratio) system.

Further purification of the antibiotic was performed chromatographically on a column of "Hyflo Supercel," according to the method of Perret(3). The effluent was collected in 18 two-ml portions, each portion neutralized with 0.006 M KOH and the radioactivity determined. With this method, 2 radioactive components were isolated (Fig. 1). The first (10th-16th ml) had no antibacterial activity, while the second (20th-28th ml) suppressed the growth of *M. pyogenes*. When known mixtures of non-radioactive phenylacetic acid and penicillin were separated by the same chromatographic method and the distribution followed titrimetrically(3), the phenylacetic acid peak corresponded to the first radioactive fraction, and the penicillin peak to the second. Evidently, the non-reacted phenylacetic acid was extracted simultaneously during the isolation of penicillin.

The amorphous material after column chromatography contained 68% purified antibiotic(3). The radioautograms of this material revealed a single spot which corresponded to benzylpenicillin. When the penicillin was hydrolyzed with H_2SO_4 as described(4) and counts were made on the resulting fractions (CO_2, phenylacetic acid and

TABLE I. Distribution of C^{14} in Fermented Beer (*Penicillium chrysogenum* BC-65, Upjohn). A 96-hour fermentation (25-27°C), shake flasks, initial pH 5.5, final 8.4, 375 μg phenylacetic acid/ml added.

	mg/160 ml	c.p.m./mg	c.p.m./mMol	c.p.m./160 ml	% recovery
Added phenylacetic acid	60.0	11.2×10^3	15.3×10^2	6.72×10^5	
Recovered penicillin	138.7	2.8×10^3	10.5×10^2	3.90×10^5	58.3
Unreacted phenylacetic acid	3.2	10.8×10^3	14.7×10^2	$.35 \times 10^5$	5.2
Harvested mycelium	1792	3.8	—	$.69 \times 10^5$	10.2
Beer residue (not extracted with amyl acetate)	—	—	—	$.03 \times 10^5$	.3
Total accounted for				4.97×10^5	74.0
Unaccounted for (CO_2, losses during the extraction, etc.)				1.75×10^5	26.0

the acidic ether-insoluble residue), only the resulting phenylacetic acid (m.p. 75-77°C) was found to be radioactive. These data indicate that the C^{14} in the phenylacetic acid used as precursor was incorporated only into the side-chain of benzylpenicillin and was not distributed throughout the whole molecule.

The specific activity of the purified penicillin (10.48×10^5 c.p.m. per mole) was lower than that of the phenylacetic acid used (15.29×10^5 c.p.m. per mole). This difference was found to be due to the dilution of the labeled antibiotic with penicillin produced from a precursor which was biosynthesized or preformed in the medium.

The distribution of radioactivity in a typical experiment, in which the described analytical methods were used is given in Table I.

The data in Table I indicate that the residual phenylacetic acid was not diluted during the fermentation, for it had practically the same specific activity as when initially added. If this acid is the normally occurring precursor of the side-chain of benzylpenicillin, it is either not released free into the medium, or is incorporated into the penicillin molecule at the same rate as it is biosynthesized.

The incorporation of phenylacetic acid into the penicillin, however, was not the only change observed when the acid was metabolized by *P. chrysogenum*. When, in similar experiments, the respiratory CO_2 was intercepted as $BaCO_3$ and counted, it was found to be radioactive, thus indicating that the acid was also decarboxylated. The data are in agreement with those recently published (5). In addition, the acid was found to be incorporated into the protein and non-saponifiable lipid fractions of the mycelium. Further work is being carried out to elaborate these diverse metabolic paths of phenylacetic acid brought about by *Penicillium*.

Summary. The biosynthesis of the C^{14}-labeled benzylpenicillin was achieved by preparing phenylacetic acid-1-C^{14} and incorporating the latter into the side-chain of benzylpenicillin. The antibiotic was successfully separated from the nonreacted labeled phenylacetic acid by column chromatography. The data obtained by radioautography and chemical degradation of the purified antibiotic indicated that the radioactivity resided in the side-chain of the benzylpenicillin molecule.

The advice and assistance of Drs. J. H. Hunter and D. H. Simonsen, Mrs. E. E. Nelson and Miss N. J. Eilers are gratefully acknowledged.

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Transplantation of a Granulosa Cell Tumor into the Spleen of Castrated Rats, Treated with Gonadotrophin.* (20581)

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Successful production of luteomas and granulosa cell tumors from implanted ovarian tissue into the spleen of ovariectomized rats was first reported by Biskind and Biskind(1,2). Luteomas appeared (at the earliest) 160 days after the implantation of ovarian tissue into the spleen, while granulosa cell tumors did not appear earlier than 300 days after the implantation. No tumors developed when the implantation was made into the kidney nor when fibrous vascular adhesions between the spleen and abdominal wall were present. The estrogen produced by the ovarian transplant in the spleen was inactivated by the liver(3), as proved by the vaginal smear. The Biskinds did not conclude which was the more important factor in the production of the tumors: the disappearance of estrogen from the circulation or the hypophyseal stimulation due to failure of the usual inhibition of the hypophysis by the estrogenic hormone. If hyperproduction of the anterior pituitary gonadotrophin played an important role, it should be possible to enhance the formation of luteomas and granulosa cell tumors by injecting gonadotrophin into the castrated and intrasplenic ovarian grafted rats. Zondek and Laufer(4) succeeded in enhancing the formation of luteomas and granulosa cell tumors in castrated rats with intrasplenic ovarian grafts, by injection of chorionic gonadotrophin, three times weekly 20-100 R.U. The tumors appeared 40-150 days earlier than in Biskind's experiments, in which the rats were not treated with gonadotrophin. The transplantation of such tumors into other animals was difficult and unsuccessful(5). Li(6) was the first to report a successful intrasplenic transplantation of a granulosa cell tumor in a mouse treated with progesterone (4 weekly injections of 1 mgr.). The original tumor developed in castrated mice with homotransplants of ovaries in the spleen, treated with subcutaneous injections of progesterone.

In the present paper the transplantation and growth of granulosa cell tumor in the spleens of castrate female rats is reported.

Methods. The original tumor was produced by repetition of Biskind's experiment (castration of rats and implantation of ovarian tissue into the spleen) without any treatment. Ten rats weighing between 110-120 g were castrated and tiny pieces of granulosa cell tumor were grafted beneath the capsule of the spleen. Three weeks later the animals were injected with 20 R.U. of chorionic gonadotrophin (Korotrin) 3 times weekly for 9 months. Of the original 10 rats, 1 died and at autopsy no tumor was found. The remaining animals were sacrificed after additional 4 months, *i.e.*, 13 months after castration and transplantation of the granulosa cell tumor.

Results. Two of them developed large splenic tumors and few or no adhesions were found around the spleen. In the other 7 rats fibrous adhesions between the spleen, surrounding organs and abdominal wall were

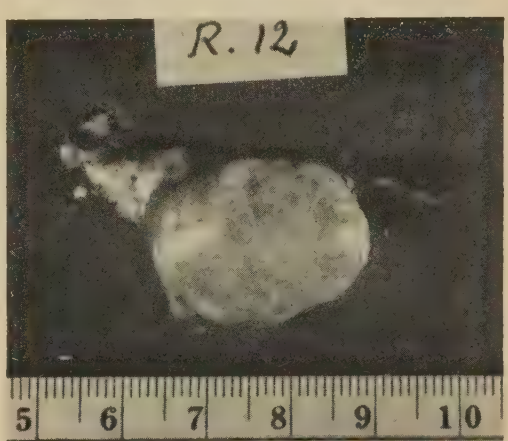

FIG. 1. Ovarian tumor, with characteristics of a granulosa cell tumor and luteoma, in the spleen.

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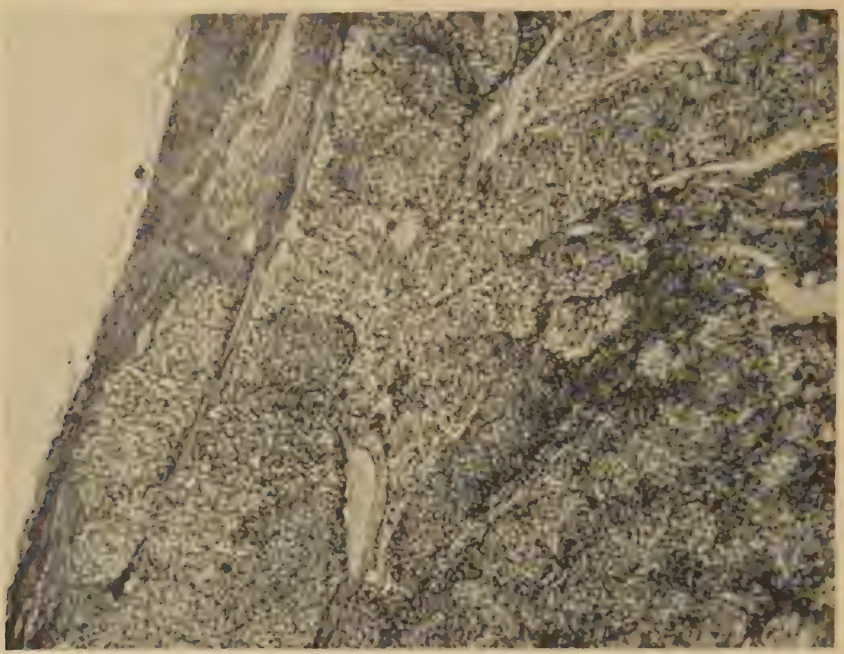

FIG. 2. Tumor nodules. Note the invading nodule into the splenic tissue, × 52.

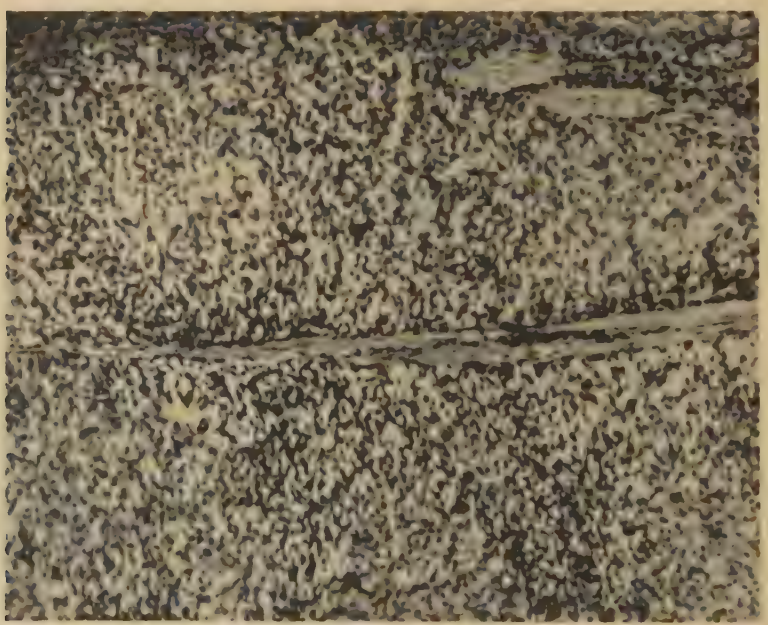

FIG. 3. Granulosa cell elements of the tumor, × 160.

numerous and vascular, and only small scars were found at the site of the transplantation.

Gross and microscopic examination of the two successful transplants revealed the characteristics of both granulosa cell tumor and luteoma. In each case the tumor was over 2 cm in diameter (Fig. 1), somewhat firm, red-yellow, lobulated, and in scattered areas showed small cysts. The tumors were in general well demarcated from surrounding splenic tissue without being encapsulated, but at one point in one of the tumors a nodule invaded

FIG. 4. Lutein elements of the tumor lining cystic spaces, × 225.

the splenic tissue (Fig. 2). Histologically there were two cell types embedded in a fine network of reticulum. One cell type corresponded to typical granulosa cells (Fig. 3) and the other was characteristic of luteum elements (Fig. 4). The two cell types were arranged in broad sheets or clusters, occasionally intermingled, and lined large cystic spaces. The tissue was well vascularized. Mitoses were easily found.

No tumor was found in 5 control rats which did not receive gonadotrophin and in which the granulosa cell tumor was transplanted into the spleen.

Conclusion and summary. The results of the present experiments indicate: 1) Ovarian tumors which develop from splenic transplanted ovarian tissue can be successfully retransplanted. 2. The retransplanted tumor resembles both a granulosa cell and luteum cell neoplasm. 3. Administration of gonadotrophin promotes the successful take. 4. It is assumed also that the prolonged stimulation by augmented amounts of gonadotrophic hormones of the hypophysis, to which the spleen grafted animals are subjected, is responsible for the development of the neoplastic growth. Additional subcutaneous injections with gonadotrophin enhance the formation of the tumors. 5. Estrogen produced by the graft must be inactivated by the liver, otherwise tumors do not develop. 6. The presence of adhesions between the spleen and surrounding tissues prevents the growth of the transplant.

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Effects of Genetics and Anesthesia Upon Granulocyte and Agranulocyte Levels in Seven Inbred Mouse Strains. (20582)

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(Introduced by C. C. Little.)

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Recent investigations of normal mouse blood(1) has shown significant genetic differences among young adults of 18 inbred strains both in total leukocytes and in proportion of granulocytes. In addition, an inverse correlation was found between mean total leukocyte count and mean proportion of granulocytes for a given strain, suggesting "that differences in genetic factors affecting production or release of lymphocytes are more prevalent or more effective than are genes affecting granulocytes." The present experiments are designed to analyze independently the numerical variations in granulocytes (largely neutrophils) and agranulocytes (largely lymphocytes) of young adults of several of these inbred strains of mice. Inasmuch as it has been suggested that differences in reactions of animals to anesthesia might affect leukocyte values, a test of the effects of nembutal anesthesia upon the level of both types of cell was also included.

Materials and methods. After thoroughly testing its applicability to mouse blood, the Randolph method for chamber enumeration of granulocytes and agranulocytes in phloxine-methylene-blue-propylene-glycol diluent(2) was utilized in these experiments. Five virgin females and 5 males, 2 to 3 months old, from seven of the most widely used of the inbred strains in the Jackson Laboratory Inbred Nucleus were used for each determination. All counts were made between 10 A.M. and 5 P.M. during August, 1952. The anesthetized animals had been injected intraperitoneally 15-20 minutes before the sample was taken with 1.5 mg nembutal in .25 cc solution. All animals were warmed in a jar under a light bulb for 10-15 minutes before samples were taken(3), and tail blood was collected after immersing the tail for 30 seconds in hot water. Two samples were taken from each animal. The mean value for each type of cell was determined for each group of animals. Analysis of variance was used to compare the variance attributable to the particular factors which can be sorted out in this experiment (strain, sex, anesthesia, and interactions among these) with the variance due to unanalyzed factors (experimental error, slight differences in nutrition or recent activity, time of day, for example) to determine whether or not the designated factors contributed significantly to the total variation. Since the groups used were small, and the tests thereby not exhaustive, only variance contributions significant at the 1% level were considered conclusive enough to be extensively discussed.

Results. Table I gives the mean values for agranulocytes and granulocytes for 10 non-anesthetized and 10 anesthetized animals from each of the strains, both with sexes separated and combined. The totals are slightly lower than earlier total leukocyte values for the same strains by the conventional acetic acid method. In general, the observed differences between strains, sexes, and conditions of anesthesia are sufficiently small so that it is impossible to determine their significance by the standard errors of individual groups of values. Agranulocyte means vary over a wider range than do granulocytes. Table II gives the results of analysis of variance of values for each type of leukocyte. Genetic differences among the inbred strains contributed very significantly to variations in both granulocytes and agranulocytes. The effects of anesthesia (in general a lowering of the count) contribute markedly to differences in granulocyte level.

* This work was done under a Grant-in-Aid to the Roscoe B. Jackson Memorial Laboratory from the American Cancer Society upon recommendation of the Committee on Growth of the National Research Council. It has also been aided by grants to the Jackson Laboratory from the Commonwealth Fund, Anna Fuller Fund, Jane Coffin Childs Memorial Fund, and the National Cancer Institute, of the National Institutes of Health, U. S. Public Health Service.

TABLE I. Mean Values (Randolph's Method) for Granulocytes and Agranulocytes of Intact and Anesthetized Animals of Each Strain Tested, with Comparison to Earlier Conventional Determinations of Total Leukocyte Counts for the Same Strains.

Strain	Sex	Agranulocytes × 10^{-3} Non-anesthetized	Agranulocytes × 10^{-3} Anesthetized	Granulocytes × 10^{-3} Non-anesthetized	Granulocytes × 10^{-3} Anesthetized	Former total leukocyte (anesth.)
						× 10^3
A/Jax	♀	5.65	6.01	1.23	.77	
	♂	5.90	3.16	1.05	.56	
	Combined	5.77	4.59	1.14	.67	8.71
AKR/Jax	♀	3.93	5.15	1.08	.84	
	♂	7.28	6.94	1.02	1.51	
	Combined	5.60	6.05	1.05	1.18	6.80
BALB/c Jax	♀	8.52	7.47	1.64	1.15	
	♂	6.92	5.26	1.56	1.28	
	Combined	7.72	6.37	1.60	1.22	8.54
C3H/Jax	♀	5.57	5.31	1.61	1.59	
	♂	5.92	2.95	2.33	1.15	
	Combined	5.75	4.13	1.97	1.37	7.34
C57BL/6 Jax	♀	7.99	8.92	.87	1.13	
	♂	8.24	6.53	.82	.89	
	Combined	8.12	7.73	.84	1.01	11.43
DBA/1 Jax	♀	7.38	6.45	1.48	1.11	
	♂	8.32	7.75	1.87	1.80	
	Combined	7.85	7.10	1.67	1.45	8.60
DBA/2 Jax	♀	7.48	5.90	1.36	.75	
	♂	7.85	6.86	1.69	1.31	
	Combined	7.66	6.38	1.53	1.03	9.28
Grand mean	♀	6.65	6.46	1.32	1.05	
	♂	7.20	5.63	1.48	1.21	
	Combined	6.92	6.05	1.40	1.13	8.41

TABLE II. Results of Analysis of Variance in Numbers of Agranulocytes and Granulocytes in Mouse Blood, with Differences in Strain, Sex and Anesthesia.

Source of variation	D.F.		Mean Sq.	F-values	Level of significance
	Agranulocytes				
Total	139				
Due to anesthesia	1	26.8669	26.8669	4.9427	.05
" " sex	1	.5982	.5982	.1101	—
" " strain	6	163.3265	27.2211	5.0078	.01
Common to sex and strain	6	78.2917	13.0486	2.4005	.05
" " strain and anesthesia	6	15.1427	2.5238	.4643	—
" " sex and anesthesia	1	16.7532	16.7532	3.0821	—
" " sex, strain and anesthesia	6	17.1878	2.8646	.5270	—
Error	112	608.7965	5.4357		
	Granulocytes				
Total	139				
Due to anesthesia	1	2.5326	2.5326	12.4208	.01
" " sex	1	.8721	.8721	4.2771	.05
" " strain	6	10.7989	1.7998	8.8269	.01
Common to sex and strain	6	2.4267	.4045	1.9838	—
" " strain and anesthesia	6	2.8113	.4686	2.2982	.05
" " sex and anesthesia	1	.0015	.0015	.0074	—
" " sex, strain and anesthesia	6	2.6105	.4351	2.1339	—
Error	112	22.8375	.2039		

It should be noted that different strains may react differently to nembutal, as factors common to strain and anesthesia produced variation (significant at the 5% level) in granulocyte number. Inspection of Table I shows that the C57BL/6 animals, in contrast to all

others, gave higher ganulocyte counts under anesthesia, and the A animals showed extreme lowering of the count with nembutal.

Discussion. These results of direct chamber count of agranulocytes and granulocytes in mouse blood have confirmed earlier findings of significant differences in total leukocyte counts among inbred strains, and have proved that there are significant differences in both types of cells, answering a question left in doubt in the earlier work. In addition, these results show that the 2 cell types vary independently of each other. The rank order in non-anesthetized animals (Table I) of mean values for agranulocytes (C57BL/6 > DBA/1 > BALB/c > DBA/2 > A > C3H > AKR) is very different from that for granulocytes (C3H > DBA/1 > BALB/c > DBA/2 > A > AKR > C57BL/6. Since the cells are produced by different tissues this is not necessarily surprising.

It might be thought that the rank order of variation for granulocytes would correspond closely with that for the erythrocytes formed in the same tissue. Taking the erythrocyte values for these strains from earlier work(1), there is a certain similarity, DBA/1 and BALB/c being high in both; however, the correlation is completely spoiled by the C3H strain, which has a very high granulocyte number but the lowest of all erythrocyte means. Separation of lymphocytic from granulocytic variation has shown no more relation between number of lymphocytes (at 2-3 months) and future potentiality for lymphatic leukemia than did the previous total and differential leukocyte counts. Thus, again it must be said that "it has not been shown that an observed strain difference is one facet of a difference in a particular known pattern of physiological balance. . . . It may equally well be that there actually is no selective advantage within the total observed range . . . (and) . . . the observed strain difference(s) might be chance fixation of a particular genotype."

The significant effect of anesthesia is of particular importance to analysis of existing literature and to planning of experiments. For example, the usefulness of the earlier work on total leukocyte counts of these same strains (1) quoted in this paper is severely limited by the fact that the total leukocyte counts were taken on anesthetized animals, while the determinations of % granulocytes were on non-anesthetized animals. The possible differing reaction of different strains to nembutal should also be of importance to the planning of experiments. Further tests of strain difference in reaction of lymphocyte and neutrophil level to nembutal anesthesia, using larger numbers of animals in each group, would be a great scientific service.

These data have definitely increased knowledge of the leukocyte characteristic of this group of inbred strains, particularly in demonstrating the reality and independence of genetic variation in granulocytes from the bone-marrow and lymphocytes from the lymphoid tissue.

Summary. 1. Direct chamber counts of granulocytes and agranulocytes were made by the Randolph method in the blood of 2-3-month-old virgin females and males of 7 inbred strains, with and without nembutal anesthesia. 2. Analysis of variance demonstrated significant genetic differences among the strains in both granulocyte and agranulocyte counts. 3. Anesthesia contributes significantly to variations in granulocyte number. 4. From these data, the observed strain differences do not appear to be related to differences in other known physiological characteristics.

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Effect of Glycerol and Freezing on Some Staining Reactions of Human Spermatozoa. (20583)

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Recently we(1) observed that human spermatozoa in glycerol treated semen showed no obvious changes in motility or morphology after surviving freezing and thawing. It was decided, however, to test for more subtle alterations which may occur within the spermatozoa as a result of such treatment. This was suggested by the observation of Keilin and Hartree(2) that freezing of spermatozoa changes the absorption spectrum of their cytochrome and Tyler's(3) report that it may increase their release of a protein lysin. Moreover, Lovelock(4) reported modifications in salt concentration during the freezing of red blood cells and indicated the general application of his results to other cells as well.

The purpose of this communication is to present the results of an experiment designed to detect possible differences between the protein of spermatozoal nuclei in untreated and glycerol treated semen before freezing and after thawing. The principle underlying the technic employed is that "staining intensity varies with changes in the protein and, consequently, is a sensitive criterion of the modification to which the protein was previously subjected." (Singer(5)).

Methods. Dyes. Seven oxidation-reduction potential indicator dyes were employed (Table I). A 0.5% solution in appropriate buffers was prepared for each dye at pH 4, 5, 6, 7, 8, 9, and 10 (when solubility permitted). Acetate buffer was used for pH 4, 5, and 6, phosphate buffer for pH 7 and borate buffer for pH 8, 9, and 10. *Semen.* Semen specimens, considered normal by the usual seminological tests, (Farris(6)), were obtained from 2 donors and processed separately. After liquefaction, a portion of the semen was treated with glycerol, 9 parts semen to one part absolute glycerol. Four 12 x 75 mm bacteriological test tubes were then prepared, 2 with 0.5 cc portions each of untreated semen and 2 with the same quantity of treated semen. One tube each of untreated and treated semen was then frozen by placing them in an insulated box containing dry ice (—70°C) and were subsequently thawed 24 hours later in a 37°C water bath. The semen from these 4 tubes was processed as follows: *Staining technic.* Smears were made on ordinary 3″ x 1″ slides and fixed overnight with equal parts of 95% alcohol and absolute ether. The slides were then washed in two 5 minute changes of absolute alcohol containing silica gel and dried for 10 minutes in a 37°C oven. The dried smears were placed in the dye solution for one minute and then dipped 5 times in first 50% then 80% alcohol to remove excess dye. This was followed by dehydrating in 2 absolute alcohol baths, one minute each, and by clearing in xylol for 2 minutes prior to mounting in a synthetic resin.

Microscopic examination. Using the same microscope and illumination, each author separately rated the intensities of nuclear staining. In some areas there were minor disagreements on intensity. These were resolved by re-examination. The intensity classification employed is shown in Table I.

Results. The results of our study are summarized in Table I and were identical for the 2 semen specimens examined. No differences in staining intensities were noted between: 1) Fresh semen, untreated and treated with glycerol, 2) Frozen-thawed semen, untreated and treated with glycerol, 3) Fresh and frozen-thawed semen, untreated and treated with glycerol.

Discussion. Knowledge of the relative staining intensities achieved with a number of oxidation-reduction potential indicator dyes at different pH ranges probably gives information concerning changes at the sites of dye binding on the proteins which are stained. Such information may also indicate changes in the isoelectric points of these proteins. However, Klotz(7,8), among others, presents chemical evidence to cast doubt upon the pre-

TABLE I. Staining Intensities of Spermatozoal Nuclei, of Untreated and Glycerol Treated Semen before Freezing and after Thawing, Following Exposure to Dyes Indicated.* Intensity is designated as 0 = unstained, ± = faint, + = light, 2+ = moderate, and 3+ = dark. I = dye insoluble at given pH.

Dye	E_0^1	pH						
	pH 7	4	5	6	7	8	9	10
Sodium 2,6, dichlorobenzenone indophenol	+.217	I	I	0	0	0	0	0
Brilliant cresyl blue	+.047	+	+	+	2+	+	0	0
Methylene blue chloride	+.011	+	+	2+	3+	3+	3+	3+
K_4 indigo tetrasulfonate	—.046	+	+	±	±	0	0	0
Cresyl violet	—.167	2+	3+	3+	3+	+	I	I
Safranin bluish	—.421	2+	2+	2+	3+	3+	3+	3+
Phenosafranin	—.525	+	2+	2+	3+	2+	3+	3+

* Since pattern of staining, with reference to various dyes at given pH's, was the same for all semen samples, a single table here represents the data obtained.

cision of measurements of either the isoelectric points or the oxidation-reduction potentials of proteins through staining technics. Our conclusions, however, concern only the detection of *relative* modifications in those proteins which are stained under the conditions of the experiment and no such *relative* alterations were detected in spermatozoal samples treated and frozen as described.

Summary. No differences were observed between the staining intensities of spermatozoal nuclei in untreated and glycerol treated semen, either before freezing or after thawing, when they were stained at pH 4, 5, 6, 7, 8, 9, or 10 with certain oxidation-reduction potential indicator dyes. On the basis of this criterion, it has been demonstrated that treating 9 parts of liquefied semen with one part absolute glycerol, placing it in an insulated box containing dry ice (—70°C) for 24 hours, and then thawing it in 37°C water bath does not modify those proteins of the spermatozoal nucleus which are stained.

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Alteration of Activity of Thyroid Gland of Beef Cattle with Testosterone.*† (20584)

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The ability of male hormones to cause weight increases in animals of several species has been reviewed by Kochakian(1). Recently the ability of testosterone to increase the average daily gain in beef cattle has been clearly demonstrated(2,3). The activity of the thyroid gland may be very important in the elucidation of the action of testosterone in stimulating growth processes.

Materials and methods. Twelve heifers and 12 steers were divided into 2 groups at random so that 6 heifers and 6 steers were in each group. One group served as controls and received no treatment while the second group was designated as the testosterone group and each calf received weekly intramuscular injections of 1 mg of testosterone per kg of body weight. The average daily gain and feed required per unit gain for individual calves were obtained for the period from 500 to 800 lb live weight and are given in Table I for the treatment and sex groups. When each calf reached 800 lb live weight it was slaughtered and the weight of the thyroid gland taken. A representative sample of the gland was fixed in Bouin's fixative and later sectioned, stained and examined for changes in histological structure. The remainder of the thyroid gland was placed in storage at –10°C. When all the animals had been slaughtered, the thyroid glands of 2 heifers and 2 steers in each treatment group were individually macerated in distilled water with a Waring blender for 5 minutes and then strained through a double layer of cheese cloth. The filtrate was diluted with distilled water until one gram of original thyroid tissue was contained in 7.5 ml. The diluted filtrate was injected into mice 10 weeks of age, which had been on a ration containing 0.1% of thiouracil for 4 weeks. One subcutaneous injection of 0.2 ml of the thyroid gland suspension was given each mouse and the mice were killed 36 hours later. Thyroid tissue from each calf was injected into 3 male and 4 female mice. The mice were asphyxiated in one-half pint jars sealed with mason lids and maintained at room temperature (24°C). The time interval required for death by asphyxiation in the sealed jars was recorded for each mouse.

Experimental. Testosterone treatment produced a significant increase in the size of the thyroid glands of both heifer and steer calves, (Table I). Histological examination of the thyroid glands revealed that, while the thyroid glands of the control calves were characterized by large colloid areas with epithelium of a low or cuboidal form, the thyroid glands of testosterone-treated calves were characterized by very small colloid areas with tall epithelial cells surrounding them. In control calves a very large amount of the total volume was

TABLE I. Effect of Testosterone on Feed-Lot Performance and Thyroid Activity of Beef Heifers and Steers.

Treatment group	Total digestible nutrients consumed (lb) Per day	Per lb gain	Avg daily gain (lb)	Wt of thyroid gland (g)	Survival time of assay mice (min.)
Control heifers	10.39	4.98	2.09	16.3	43.2
Testosterone-treated heifers	9.89	3.79*	2.61*	19.4*	48.1*
Control steers	10.48	3.98	2.65	17.2	45.6
Testosterone-treated steers	10.05	3.69*	2.74*	19.6*	48.4*

* Indicates a significant difference from corresponding control (.05 level).

* This study was conducted in cooperation with Bureau of Animal Industry, U. S. Department of Agriculture, and State Experiment Stations under Western Regional Project W-1 on Beef Cattle Breeding Research.

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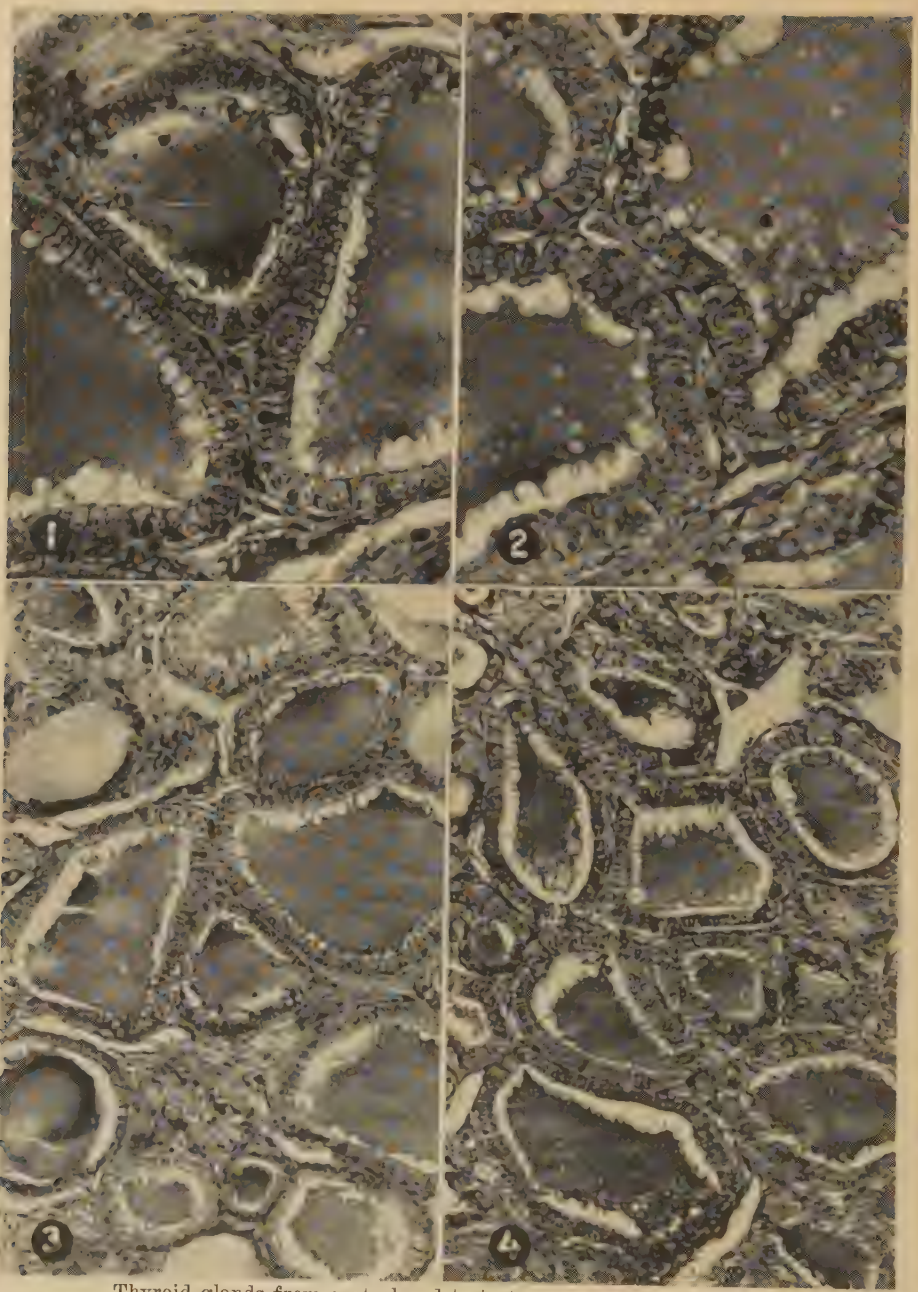

Thyroid glands from control and testosterone-treated beef cattle.

FIG. 1. Thyroid from control animal. × 240.

FIG. 2. Thyroid from animal receiving injections of testosterone. Note increased height of epithelium of follicle compared with control (Fig. 1). × 240.

FIG. 3. Thyroid from control animal. Note large amount of colloid in follicles. × 120.

FIG. 4. Thyroid from animal receiving testosterone injections. Note reduction in colloid and greater proportion of cellular material. × 120.

occupied by colloid while in the testosterone-treated calves less than one-fourth of the total volume of the gland was occupied by colloid (plate 1).

Testosterone had a definite effect on the ability of the thyroid gland to alter the time

TABLE II. Effect of Testosterone Treatment upon Ability of Thyroid Gland Tissue from Experimental Calves to Decrease the Time Required for Asphyxiation of Thiouracil-Treated Mice.

Source of thyroid gland tissue	Sex of mice used		
	Male	Female	All mice
	Survival time (min.)		
Heifers			
Control	33.6	50.4	43.2
Testosterone	36.0	57.1	48.1
Steers			
Control	35.3	53.3	45.6
Testosterone	37.0	56.9	48.4

required for hypothyroid mice to die of asphyxiation. As is indicated in Table II, the mice receiving thyroid material from control calves asphyxiated more quickly than those receiving thyroid tissue from testosterone-treated calves. In both cases the time required for asphyxiation was less than that of mice receiving no thyroid tissue.

Discussion. The increased average daily gain and feed efficiency of the calves receiving testosterone treatment as compared with control calves appears to be associated with an increased size of the thyroid gland and increased activity. The appearance of the thyroid gland of testosterone-treated calves is indicative of a highly active state. The stores of thyroxine in the colloid areas have been depleted and the epithelial cells surrounding these colloid areas are tall columnar in appearance indicating a high degree of secretory activity. The inability of thyroid tissue of testosterone-treated calves to decrease the asphyxiation time of the thiouracil-treated mice as much as thyroid tissue from control calves is indicative of a lower concentration of thyroxine in the gland and as a result a somewhat slighter effect on metabolic activity and asphyxiation time. This is an excellent example of an endocrine gland in which the retained hormone concentration is not directly proportional to the activity of the gland in secreting that hormone.

Summary and conclusions. 1. Testosterone injected intramuscularly at the rate 1 mg/kg of body wt per week in beef heifers and steers from 500 to 800 lb live wt had the following effects: a) Increased average daily gain and decreased feed requirements per unit gain. b) Increased weight of the thyroid glands. c) Increased secretory activity of the thyroid gland. d) Decreased stores of thyroxine in the thyroid gland. 2. Correlations between thyroid gland activity and growth rate are suggested as indicating a possible mode of the action of testosterone in affecting growth of beef cattle. 3. A method is described using the time of asphyxiation of thiouracil-treated mice as a measure of thyroxine content of the thyroid gland.

The authors are grateful to the Schering Corp., Bloomfield, N. J., who supplied the hormones used in these experiments.

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Mixed Infections with Coxsackie and Lansing Poliomyelitis Viruses in Mice.* (20585)

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It is already known that under certain circumstances 2 viruses may propagate simultaneously in one host and in the same organ or tissue, each presumably utilizing individual metabolic pathways(1-4). Numerous studies have also shown that under certain experimental conditions one virus may exert an interfering or sparing effect on the other(5-7), or one agent may exalt the virulence of another(8-10). This preliminary report is concerned with certain reciprocal effects observed in mice infected with both Coxsackie and poliomyelitis viruses.

Materials and methods. The 2 group B C virus strains,‡ Ohio-R (type 2) and Nancy (type 3), used in these experiments, have been maintained by passage in 2-day-old Swiss albino mice and the Lansing poliomyelitis virus,§ has been maintained by passage in adult white Swiss mice. The infectivity titer of the stock Lansing virus prepared from cords and medullae from animals which succumbed to the infection within 5 days was $10^{-3.8}$ LD_{50}. Each stock C virus suspension consisted of 20% brain or carcass from infant mice which had become paralyzed following subcutaneous inoculation. The quantity of virus present was determined by subcutaneous inoculation of randomized 2-day-old suckling mice and the 50% end-point (LD_{50}) was calculated according to the method of Reed and Muench (11). When the intraspinal route of inoculation was used the technic of Habel and Li(12) was employed. Animals which died or were found dead within 24 hours after inoculation of the Lansing virus were excluded from the analyses and were considered to be deaths from trauma.

All animals were observed twice and sometimes 3 times daily during the observation period of 30 days for extent of paralysis and deaths from poliomyelitis or C virus infection. Swiss albino mice (CFW) were used throughout these studies.

Experiments with the Ohio-R C virus and the Lansing Virus. Each animal in one group of seventy-nine 4-week-old mice received one intracerebral inoculation of 10,000 LD_{50} of C virus 5 days prior to an injection of the Lansing virus (150 LD_{50}). The 5-day interval used in this experiment is presumed to represent the time required for the blocking virus (Ohio-R) to propagate to the necessary level in the tissue of predilection of the virus which is to be excluded. Normal infant mouse carcass tissue was substituted for C virus in a second group of 81 mice. Each animal in a third group of 30 mice received an injection of C virus followed 5 days later with an injection of normal adult mouse brain tissue. Brain-cord suspensions from animals which died without previous evidence of paralysis and which had received both viruses were tested for C virus by intraperitoneal inoculation of 2-day-old mice and for Lansing virus by intracerebral inoculation of 3-week-old mice.

The results summarized in Table I show that this strain of C virus influenced significantly both the course and outcome of the experimental Lansing infection. The sparing effect became evident within 4 days after injection of the Lansing virus and persisted throughout the course of the experiment. When the experiment was terminated after an observation period of 30 days less than 10% of the controls which had received Lansing virus alone had survived as compared with 36.7% of those which had been inoculated with both viruses. This difference is highly

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‡ Kindly provided by Dr. Joseph L. Melnick.

§ Obtained from Dr. Albert Milzer.

TABLE I. Mixed Infections with Coxsackie Virus (Ohio-R Strain) and Lansing Poliomyelitis Virus in Adult Mice.*

No. of mice		79				81		
Procedure		C virus (Ohio-R) and Lansing virus†				Normal infant mouse carcass tissue and Lansing virus‡		
		Polio deaths		Deaths without paralysis§		Polio deaths		
Results		No.	Following paralysis	Polio deaths	C virus deaths	No.	Following paralysis	Without paralysis
Animals infected and dying from poliomyelitis or C virus infection (days after inj. of Lansing virus)	3	2 (2.5)‖		2	1	1 (1.2)		1
	4	8 (12.6)	5	3	1¶	19 (24.7)	17	2
	5	7 (21.5)	6	1		18 (46.9)	18	
	6	3 (25.3)	2	1	1	6 (54.3)	5	1
	7	7 (34.2)	6	1	1**	4 (58.3)	4	
	8	6 (41.8)	4	2	1	0 (58.3)		
	9	3 (45.5)	3			4 (64.2)	2	2
	10	2 (49.9)	1	1		4 (69.2)	3	1
	11-13	5 (53.1)	4	1		10 (81.4)	9	1
	14-16	4 (59.5)	3	1		5 (88.6)	5	
	17-20	2 (62.0)	1	1		2 (90.1)	2	
	21-25	1 (63.3)	1			0 (90.1)		
	26-30	0 (63.3)				0 (90.1)		
Survivors		36.7%				9.9%		

* Unless otherwise indicated all inoculations were made i.c.; inoculum 0.03 ml.
† One inj. of C virus (10000 LD_{50}) 5 days prior to an inj. of Lansing virus (150 LD_{50}).
‡ " " " normal infant mouse carcass (0.03 ml of a 1.0% suspension) instead of C virus.
§ Brain and cord tested for C virus in suckling mice (i.p.) and Lansing virus in 3-wk-old mice (i.c.).
‖ Figures in parentheses indicate cumulative % deaths.
¶ Both Lansing and C virus demonstrated in this brain-cord suspension.
** C virus LD_{50} titer $10^{-4.8}$.

significant ($p = <.0001$). Similar results with the Conn.-5 and Nancy strains of group B C viruses have been observed by Dalldorf(6). More recently, Stanley(6) reported that certain group B C viruses isolated in Sydney, Australia prolonged the incubation period of MEF_1 poliomyelitis virus infection in adult mice.

On the basis of tests for both viruses there was evidence that Ohio-R strain proliferated in the CNS of some of the animals which had also received Lansing virus. At least 5 of the deaths without previous evidence of paralysis were considered to be due to C virus because a 10^{-3} brain-cord suspension produced characteristic C virus infection in suckling mice following intraperitoneal inoculation and failed to produce infection in adult mice following intracerebral inoculation. In one instance the C virus LD_{50} titer was $10^{-4.8}$ and in another both Lansing and C viruses were demonstrated in the CNS. In many instances it was possible to distinguish between the two experimental infections; the symptoms resulting from C virus infection consist of roughened coat, hunching, tremors and spasticity, while in the case of Lansing infections, aside from flaccid paralysis of one or more limbs, the animals have a smooth coat and appear healthy. None of the 30 adult mice which had received massive doses of C virus followed by injections of normal mouse brain showed evidence of virus proliferation in the CNS. Aside from the evidence, then, that the Ohio-R strain of C virus exerts a sparing effect in adult mice infected with Lansing virus, the latter seems to enhance the virulence of this C virus for these otherwise insusceptible animals. Experiments to determine the extent to which Lansing and other infections in adult mice increases the susceptibility in such animals to C virus infections are still in progress.

Experiments with the Nancy virus and the Lansing virus. All inoculations in this experiment were made intracerebrally. Each animal in one group of forty-four 4-week-old mice received one injection of 500,000 LD_{50} of Nancy virus 5 days prior to an injection of approximately 10 LD_{50} of Lansing virus. The animals in a second group received normal

TABLE II. Effect of Coxsackie Virus (Nancy Strain) on Course and Incidence of Disease in Adult Mice Infected with Lansing Poliomyelitis Virus.*

Procedure	C virus (Nancy) and Lansing virus†				Normal infant mouse carcass tissue and Lansing virus‡			
		Limb paralyzed first				Limb paralyzed first		
Results	No.	Fore-limb	Hind-limb	Without paralysis	No.	Fore-limb	Hind-limb	Without paralysis
Exp. 1	44 mice				70 mice			
Animals infected and dying from poliomyelitis (days after inj. of Lansing virus) 3					2 (2.8)	1		1
4					6 (11.4)	5		1
5	2 (4.5)§		2		7 (21.4)	6	1	
6	2 (9.1)	1	1		1 (22.8)	1		
7	2 (16.6)	1	1		2 (25.6)	2		
8	3 (20.5)	2	1		2 (28.5)	2		
9	1 (22.5)		1		4 (34.2)	3	1	
10-13	1 (25.0)		1		2 (37.1)	1		1
14-17	2 (29.5)	1		1	6 (45.7)	5		1
18-30	0 (29.5)				0 (45.7)			
Survivors	70.5%				54.3%			
Limb paralyzed first¶								
Forelimb	41.7%				92.9%			
Hindlimb	58.3%				7.1%			
Ratio Forelimb/Hindlimb	0.7				13			
Exp. 2	56 mice‖				51 mice			
Animals infected and dying from poliomyelitis (days after inj. of Lansing virus) 3					1 (1.9)			1
4	5 (8.9)	4	1		13 (27.4)	9	3	1
5	8 (23.2)	3	5		8 (43.1)	8		
6	6 (33.9)	1	5		4 (50.9)	3		1
7	9 (50.0)	6	3		1 (52.9)	1		
8	4 (57.1)	3	1		0 (52.9)			
9	5 (66.1)	4	1		2 (56.8)			2
10-13	1 (67.9)	1			11 (78.4)	9		2
14-17	1 (69.6)	1			4 (86.2)	3	1	
18-30	0 (69.6)				0 (86.2)			
Survivors	30.4%				13.8%			
Limb paralyzed first¶								
Forelimb	58.9%				90.0%			
Hindlimb	41.1%				10.0%			
Ratio Forelimb/Hindlimb	1.4				8.2			

* All inoculations were made i.c.; inoculum 0.03 ml.
† One inj. of C virus (500000 LD_{50}) 5 days prior to an inj. of Lansing virus (10 LD_{50}).
‡ One inj. of normal infant mouse carcass tissue (0.03 ml of a 1.0% suspension) instead of C virus.
§ Figures in parentheses indicate cumulative % deaths.
‖ 150 instead of 10 LD_{50} of Lansing virus.
¶ Percentages calculated on the basis of those that died following paralysis.

infant mouse carcass tissue instead of C virus and served as controls. Each of 25 mice in still another group received massive doses of C virus followed by an injection of normal adult mouse brain tissue. The results of this experiment summarized in Table II (Exp. 1) indicate that the sparing effect which was evident early in the course of this experiment became less conspicuous as the experiment progressed. When the study was completed the difference in survival rates among controls as compared with those which had received both viruses was not statistically significant. None of the animals which had received large doses of C virus followed by normal mouse brain showed evidence of infection, and the Lansing virus failed to alter the susceptibility of the adult mice to the Nancy infection. The Nancy virus, however, seemed to have a noticeable effect on the pathogenesis of the Lansing virus infection. Generally, forelimb paralysis occurs first in a large proportion of animals inoculated with Lansing virus(13,14). In these studies 93% of the animals receiving

TABLE III. Influence of Route of Inoculation of Coxsackie Virus (Nancy Strain) on Course and Incidence of Disease in Adult Mice Infected with Lansing Poliomyelitis Virus.

	No. of mice	68				56			
	Procedure	C virus (Nancy) and Lansing virus*				Normal infant mouse carcass tissue and Lansing virus†			
		Limb paralyzed first				Limb paralyzed first			
	Results	No.	Fore-limb	Hind-limb	Without paralysis	No.	Fore-limb	Hind-limb	Without paralysis
Animals infected and dying from poliomyelitis (days after inj. of Lansing virus)	3	4 (5.9)‡			4	2 (3.6)			2
	4	2 (8.9)			2	2 (7.2)	2		
	5	8 (20.6)	4	2	2	6 (18.0)	4	2	
	6	8 (32.3)	8			4 (25.2)	4		
	7	2 (35.3)	2§			2 (28.6)	2		
	8	0 (35.3)				2 (32.2)	2		
	9	2 (38.2)		2		2 (35.9)	2		
	10-13	10 (52.9)	10			10 (53.6)	10		
	14-20	8 (64.7)	2	2	4	16 (82.2)	16		
	21-30	8 (76.4)	8			10 (100.0)	10		
Survivors		23.6%				0			
Limb paralyzed first‖									
Forelimb		85.0%				96.3%			
Hindlimb		15.0%				3.7%			
Ratio Forelimb/Hindlimb		5.7				26			

* One i.s. inj. of C virus (500000 LD_{50}) 6 days prior to i.c. inj. of 150 LD_{50} of Lansing virus.

† One i.c. inj. of 150 LD_{50} Lansing virus 6 days after i.s. inj. of normal infant mouse carcass tissue (0.03 ml of 1.0% suspension).

‡ Figures in parentheses indicate cumulative % deaths.

§ One of these animals survived 15 days after first evidence of paralysis.

‖ Percentages calculated on the basis of those that died following paralysis.

Lansing virus alone developed forelimb paralysis first, while this occurred in only 41.7% of those which had received both viruses. In the group of animals which had received both viruses the frequency with which the forelimbs became paralyzed first as compared with the frequency of hindlimb paralysis appearing first showed a ratio of 0.7 to 1. In the control group this ratio was 13 to 1. It is conceivable that the C virus exerts its effect in the higher level of the CNS by "forcing" the Lansing virus to descend the spinal cord to those areas where innervation of hindlimb muscles arises. When this experiment was repeated at a later date with a somewhat larger challenge dose of Lansing virus (150 LD_{50}) essentially the same results were obtained (Table II, Exp. 2).

Another experiment was designed to determine the influence of route of inoculation of the Nancy virus on the pathogenesis of the Lansing virus infection. Each adult mouse in one group received one intraspinal injection of Nancy virus (500,000 LD_{50}) 6 days prior to an intracerebral injection of 150 LD_{50} of Lansing virus. Normal mouse carcass tissue was substituted for C virus in another group of mice, while still another group of mice received an intraspinal injection of C virus followed by an intracerebral injection of normal mouse brain tissue. The results of this experiment are summarized in Table III. No blocking effect was evident for at least 15 days following the injection of Lansing virus. When the experiment was terminated, however, none of the controls survived while 23.6% of those animals which had received both viruses survived; a difference which is statistically significant ($p = .006$). Calculated on the basis of those which died following the development of paralysis, over 96% of the animals receiving Lansing virus alone developed forelimb paralysis first, while this occurred in 85% of those which had received both viruses. In the control group the frequency with which the forelimbs became paralyzed first as compared with the first appearance of hindlimb paralysis showed a ratio of 26 to 1. In the group which had received both viruses this ratio was 6 to 1. Not detailed in the tabulation is the fact that the progress of the infection in the control group followed the usual course with development of hindlimb paralysis in a large

proportion of animals prior to death. Many of the animals which had received a previous intraspinal injection of C virus failed to develop hindlimb paralysis prior to death. This would suggest that the presence of C virus in the lumbar region of the spinal cord limits extension of the Lansing virus thus altering the normal progress of this experimental infection. Experiments designed to determine the validity of this concept are in progress. None of the adult mice which had received an intraspinal inoculation of C virus followed by an intracerebral injection of normal brain tissue showed evidence of virus proliferation. There was no evidence of exaltation of the Nancy strain as demonstrated previously with the Ohio-R virus. In addition to influencing the survival rate of Lansing infection in mice, the intraspinal inoculation of the Nancy virus had a definite effect on the survival time after paralysis was first observed. Animals which had received both viruses survived, on the average, twice as long as those which had received Lansing virus alone. In one instance an animal which had received both viruses developed forelimb paralysis 7 days after the injection of the Lansing virus and died 15 days later. Another animal which had received both viruses developed forelimb paralysis 22 days after the injection of the Lansing virus and was sacrificed over 6 months later.

In addition to the extreme variability in the interval between the intracerebral inoculation of the Lansing poliomyelitis virus and the onset of paralysis, a biphasic distribution of incubation periods occurs with some regularity (14-16). In the present experiments‖ the biphasic distribution of incubation periods was evident only among those animals which had received Lansing virus alone. Neither of the groups of mice which had received intracerebral injections of the Ohio-R or Nancy viruses prior to the Lansing inoculation showed evidence of biphasic distribution of incubation periods.

Summary. The following reciprocal effects have been observed in mice infected with viruses which do not possess the same degree of pathogenicity for the host. 1. The Ohio-R strain of C virus influenced significantly both the course and outcome of experimental Lansing poliomyelitis infections in 4-week-old white Swiss mice. 2. In the presence of the Lansing infection the virulence of the Ohio-R virus seemed to be enhanced for these otherwise insusceptible animals. 3. The Nancy strain of C virus had a noticeable effect on the pathogenesis of the Lansing infection in 4-week-old mice. The frequency with which the forelimbs became paralyzed first as compared with the first appearance of hindlimb paralysis was influenced by the presence of Nancy virus in the higher levels of the CNS. The presence of Nancy virus in the lumbar region of the spinal cord, following intraspinal inoculation, seemed to limit extension of the Lansing virus thus altering the normal progress of this experimental infection. 4. There was no evidence of enhancement of the virulence of the Nancy virus in the presence of the Lansing infection. 5. In addition to exerting a sparing effect on the Lansing infection, animals which had received a previous intraspinal inoculation of Nancy virus survived twice as long as those which had received Lansing virus alone. 6. The biphasic distribution of incubation periods generally observed among animals infected with the Lansing virus was not evident among those animals which had received both viruses.

‖ CFW mice were used in the experiments because they show less variability than other Swiss mice in their reaction to the Lansing virus(17).

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Comparative Protective Effect of Cysteine against Fast Neutron and Gamma Irradiation in Mice. (20586)

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There are numerous examples of protective effects by hypoxia and chemicals in radiation biology(1) and, for the most part, one is prone to interpret these phenomena in terms of the postulated energy transformations in irradiated water. Perhaps the most cogent evidence for this sort of mechanism is derived from comparison of the modifying influence of oxygen and of various substrates on the radiation-induced reactions in simple aqueous solutions and in living systems. The observations of Thoday and Read(2,3) and, more recently, of Giles *et al.*(4) are particularly significant. The former found a negligible effect of oxygen deprivation on growth reduction and chromosome aberrations in *Vicia faba* roots irradiated with alpha particles, while the latter observed an intermediate protective effect of hypoxia on aberration frequency in *Tradescantia* inflorescences exposed to fast neutrons. In each case, oxygen deprivation afforded considerable protection against the effects of gamma or X-irradiation. These findings parallel certain of the radiochemical reactions involving oxygen in aqueous solution and will be discussed in connection with the data presented here. The present experiments are concerned with a comparison of the protective efficiency of cysteine against the acute lethal effects of fast neutron and gamma irradiation in mice. Fast neutrons have been estimated to be some 4.5 times as effective as gamma rays for acute killing of mice(5). In view of the radiobiological importance of ionization density and of its contribution to chemical effects in water, useful information regarding radiation mechanisms may be gained from such comparisons of protective efficiency.

Methods. Female mice (CF1) 10 to 12 weeks of age and weighing 18-25 g were maintained on a diet of Rockland checkers and water *ad libitum*. Control and experimental animals were irradiated simultaneously, caged together in groups of 10 to 15 and otherwise handled similarly. Cysteine was administered as a 12.5% solution of the hydrochloride neutralized to pH 7 with 10 N sodium hydroxide. Only freshly prepared solutions were employed. The cysteine dose was 1200 mg/kg of body weight and was given intravenously. Control mice were injected with an equivalent volume of 5% sodium chloride. In general, cysteine was injected between 5 and 15 minutes before exposure. The exposure time varied from 80 to 90 minutes for gamma rays and 60 to 90 minutes for neutrons. The mice were exposed in the gamma-neutron radiation chamber recently described(6). The dose rate of gamma rays from the Co^{60} source was about 11-12 r per minute. Fast neutrons were produced by allowing the neutrons from the thermal column of the Argonne CP-3' reactor to impinge on uranium. Appropriate shielding devices prevented slow neutrons from reaching the animal exposure position. Several methods of dosimetry indicate that the gamma

TABLE I. Comparative Protective Effect of Cysteine.

Gamma irradiation						Fast neutron irradiation					
	Sodium chloride		Cysteine				Sodium chloride		Cysteine		
Dose, r	No. mice	% mortality 30 days	No. mice	% mortality 30 days	% dose reduction	Dose, rep	No. mice	% mortality 30 days	No. mice	% mortality 30 days	% dose reduction
961	24	50	24	12	15.7	197	21	33	21	14	8.3
968	40	75	40	45	9.8	205	35	46	36	25	6.8
992	23	61	23	17	15.9	212	15	53	15	33	6.2
1007	27	67	27	41	8.4	215	36	61	36	44	5.1
1020	30	90	30	10	29.1	239	39	85	39	64	7.3
1027	50	76	50	36	13.0	252	29	100	30	63	9.4
1054	30	77	30	40	12.0						
Mean, all groups		72		30	14.9±2.5*			66		43	7.2±.6

* Refers to stand. error of the mean.

All differences between sodium chloride and cysteine treated mice are significant ($p < .01$). Difference between mean % dose reduction for gamma and neutron irradiation is significant ($p < .05$).

contamination of the fast neutron beam is less than 10%. The dose rate of fast neutrons was 2-4 rep per minute. Animals were exposed in lucite cages previously standardized as to isodose groups for both gamma and neutron radiation. Equal numbers of cysteine and saline injected mice were included in each isodose group.

Results. The results, summarized in Table I, reveal that cysteine pretreatment affords significant protection ($p < .01$; t test) against the acute lethal effects of both fast neutron and gamma irradiation. The protection observed with fast neutrons is, however, about half of that seen with gamma irradiation. This difference in protective capacity is statistically significant ($p < .05$). When these effects are evaluated in terms of the dose-mortality curves for the two radiations, the dose reduction is 14.9% for gamma rays and 7.2% for fast neutrons. Cysteine appears to protect equally over the entire spectrum of mortality during the 30-day post-irradiation period. These findings, presented as the probability of death during each 3-day interval after exposure, are shown in Fig. 1. It may be noted that the maximum killing occurs around the 8th day with neutrons and 14th day with gamma rays. In this analysis, the individual experiments in a particular group, *e.g.*, sodium chloride-gamma irradiated or cysteine gamma-irradiated, were combined even though the radiation dosage differed from one experiment to another.

In the case of gamma irradiation there is little difference in the mean day of death over the dosage range employed. With neutrons, however, survival time decreases appreciably as the dosage is increased. In view of this dose dependence, it is necessary to examine the neutron experiments individually. The data in Fig. 2 indicate that the mean survival time (survivors counted as 30 days) is the same function of the 30-day

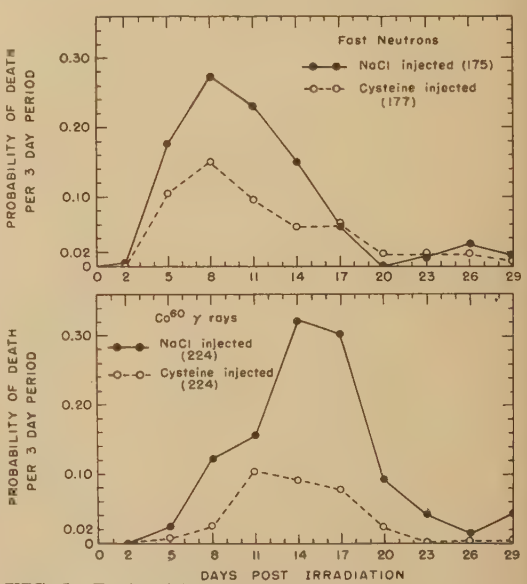

FIG. 1. Probability of death estimated over 3 day periods after irradiation of CF1 female mice with fast neutrons and Co^{60} gamma rays. (Estimates based on numbers alive at start of each interval. The figures in parentheses refer to No. of animals exposed.)

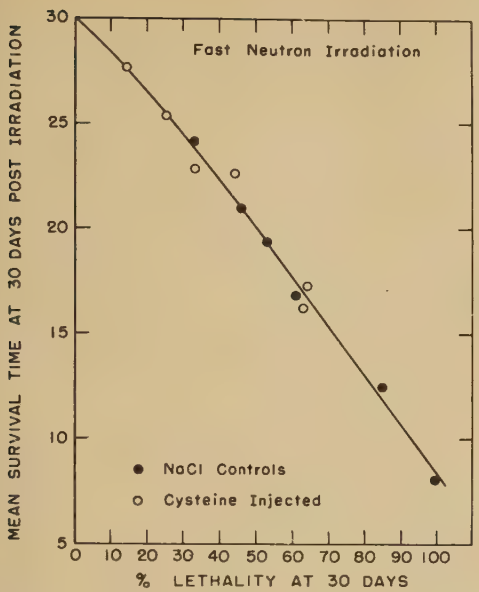

FIG. 2. Relationship between mean survival time and 30 day lethality. (Includes decedents and survivors, the latter counted as 30 days.)

mortality in both control and cysteine treated irradiated mice. This may be taken as further evidence of the general reduction in the effective neutron dose by cysteine rather than of selective protection of specific physiological systems.

Discussion. Cysteine has been shown to diminish many of the effects of X-irradiation in a variety of biological systems(1). The present experiments establish a protective effect against acute lethality in mice resulting from either gamma or fast neutron irradiation. It is noteworthy that the protection in this instance is considerably less than that reported previously for mice and rats after X-irradiation(7,8). In the case of gamma rays this may be attributed to the protracted exposure necessary to deliver a lethal dose with the facilities available. It will be recalled that the magnitude of the protection is a function of the interval between injection and irradiation as well as of the cysteine dose(7-9). A similar argument may be advanced for the neutron exposure, although this is only part of the story since the dose reduction is less for neutron than for gamma irradiation under identical conditions.

It may be noted that cysteine affords a reasonably uniform protection against a number of X-radiation sequelae in the mouse and that the efficiency of protection is apparently independent of radiation dosage(8). In general, the data presented for fast neutron and gamma irradiation are consistent with the concept of a true dose reduction in the sense that certain primary mechanisms are involved. Of particular significance is the finding that cysteine changes the mean survival time after neutron irradiation in the same manner as an equivalent reduction in radiation dosage. It follows, therefore, that the decisive protective action occurs at a common and presumably early stage in the sequence of events induced by irradiation. For the present, the most attractive mechanism involves an effect on the pathways of energy dissipation.

Since water must serve as the major repository of the initial energy transfer, it is reasonable to look for an effect on the reactions of irradiated water. Although our understanding in this area is incomplete, several salient facts may be noted: 1) irradiated water constitutes an oxidation-reduction system; 2) the number of altered water molecules per unit of energy absorbed is independent of ionization density; 3) the nature and fate of the breakdown products are dependent on ionization density, the presence of oxygen and other solutes. The high ion density radiations are generally less effective than the low ion density radiations in initiating chemical reactions in dilute aqueous solutions, whereas the reverse is true for many biological effects (10). It will be recalled that the protective effect of hypoxia decreases from gamma and X-rays to fast neutrons and is slight with alpha particles(2-4). Since the peroxide yield becomes less dependent upon oxygen with increase in ionization density, these results are suggestive of a greater role of H_2O_2 than of radical reactions *in vivo*. The greater dose rate dependence for most biological effects resulting from low ion density irradiation also parallels the situation for hydrogen peroxide formation in water(11). Other interpretations are possible, however, particularly when the matter of biological organization is considered.

In contrast to the picture in dilute aqueous

solution, it seems likely that OH radicals formed close together will have greater opportunities for reaction in the nonhomogeneous cell system with varying gradients of concentration and solubility and numerous interfaces exceeding the dimensions of a single compact radical track. Under these conditions, one might anticipate that the forward reaction to H_2O_2 would be decreased and that the radical reaction with targets in the immediate vicinity would be enhanced. The small or negligible oxygen effect with high ion density radiations is consistent with such a scheme. Since the OH radicals generated by gamma and X-rays are distributed over a larger area, these radiations should be less effective particularly when two or more radical reactions must occur in close proximity in space and time as, for example, in the production of chromosome interchanges. Hence, many types of effects with low ion density radiations may require radicals formed along two or more particle tracks, while a single particle track will suffice with high ion density radiations. The chances for interaction of hydrogen with oxygen to form HO_2 should be considerably greater with low ion density radiations, the result being an increase in effective radical concentration in the presence of oxygen with a corresponding increase in biological efficiency. The greater dose rate dependence of gamma and X-ray than that of neutron and alpha ray effects may also be interpreted in terms of an increase in effective radical concentration without regard to whether this is brought about by OH, HO_2 or H_2O_2.

The experimental findings in general are consistent with the assumption that cysteine serves as absorbing material for the oxidants formed in intracellular water. The decreased effectiveness with fast neutrons in contrast to gamma rays may then be attributed to a greater disparity in the concentration of radicals and peroxide along the particle tracks relative to that of cysteine. However, this interpretation is perhaps too naive for the biological system. Moreover, there is independent evidence that suggests that cysteine may act at least in part by diminishing the availability of oxygen(12-14). In this connection it may be noted that biological additivity of two briefly spaced X-ray doses in mice is a simple function of the cysteine dose preceding each exposure(8) and that a similar additivity obtains for the oxygen effect on broad bean roots(15). Differences in the magnitude of protection by cysteine against the effects of gamma and fast neutron irradiation in mice are in essential agreement with the inverse relationship between ionization density and the extent of the oxygen effect. Some oxygen effect would be anticipated with fast neutrons, its magnitude depending on the energy of the recoil protons.

It should be emphasized that there is no compelling reason to think of this sort of chemical protection only in terms of some interaction with, or interference in the production of, one or another of the primary radiotoxins derived from water. Protection of thymic cells by cysteine is dependent upon temperature both before and during the early post X-irradiation period(16). Thymic cells (14) and onion epidermis cells(17) are also partially protected when the amino acid is added immediately after X-irradiation. These findings suggest that at least some of the protective phenomena cannot be interpreted completely in terms of immediate oxidative reactions. It is necessary, therefore, to inquire about possible cysteine effects on the physico-chemical relationships of cell constituents and on other more delayed or secondary reactions as well. Such effects may be relatively less important with high ion density radiations owing to the distinctive distribution of the reactive intermediates.

Summary. Cysteine pretreatment has been shown to confer significant protection against the acute lethal effects of gamma (Co^{60}) and fast neutron (fission) irradiation in mice. The protection observed with fast neutrons is, however, about half of that found with gamma irradiation. The data presented support the concept of a true dose reduction in the sense that primary mechanisms are involved. The inverse relationship between protective efficiency and ionization density is comparable to that noted for the oxygen effect and, for the present, may best be interpreted in terms of

the spatial distribution of the radicals formed in water and their reactions.

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Effect of Carbonic Anhydrase Inhibitor (6063) on Arterial-Alveolar CO_2 Gradient in Man.* (20587)

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Inhibitors of carbonic anhydrase activity have been employed in the effort to block the *in vivo* reaction, $H_2O + CO_2 \rightleftharpoons H_2CO_3$. Various physiological mechanisms are dependent upon this reaction. Höber(1) first demonstrated that sulfonilamide, a carbonic anhydrase inhibitor, impairs the acidification mechanisms of the frog kidney. The use in the dog of stronger sulfonamide inhibitors, especially Diamox (6063 or 2-acetylamino-1, 3,4-thiadiazole-5-sulfonamide), has implicated carbonic anhydrase in the secretion of hydrochloric acid by the stomach(2) and of bicarbonate by the pancreas(3). It has been recently shown(4) that Diamox increases plasma bicarbonate concentration and pCO_2 in marine fishes, an effect possibly attributable to inhibition of erythrocyte carbonic anhydrase.

Roughton(5) showed that carbon dioxide exchange in unanesthetized humans was impeded when sulfonilamide was combined with exhaustive exercise. In anesthetized dogs(6), Diamox is reported to interfere with CO_2 transport, possibly by inhibition of the erythrocyte enzyme system. It was the purpose of this study to determine whether the carbonic anhydrase inhibitor, Diamox, in concentrations adequate to inhibit renal acid excretion, would interfere with the transport of CO_2 from pulmonary capillary blood into the alveoli in normal, non-anesthetized human subjects.

The subjects were 3 convalescent patients without cardiac, renal, or pulmonary diseases from the First (Columbia University) Medical Division of Bellevue Hospital. These pa-

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TABLE I. Effects of Diamox (6063) on Resting Man.

	Time, min.	V, ml/min.	Urine Bicarbonate, mM/L	$U_{Na}V$, μEq/min.	U_KV, μEq/min.	pH	C_{CR}	Respiratory gases V_E, M^2/BSA	$P_A CO_2$, mm Hg	RQ	Arterial blood pH	Pa CO_2, mm Hg	Total CO_2, mM/L
Subject A:	0	.9		229	89	7.41	128	4.12	43	.89	7.39	46	24.2
Control	10	.7	39.1			7.35*		3.98	41	.87	7.39	45	24.2
Subject A:	30												
After Diamox	72	5.3		638	244	7.75	137						
	83	13.7		700	288	7.66							
	86	15.3	33	596	231	7.30*	130						
	100	14.0		567	213	7.47							
	117	8.3		590	222	7.64	130						
	125	5.6	57	586	180	7.51*	134	4.02	40	.81	7.37	42	21.5
	160	2.9				7.74	134	4.01	38	.76	7.35	40	20.5
Subject B:	0							3.67	39	.83	7.40	40	23.8
Control	12							3.77	40	.81	7.41	41	23.2
Subject B:	120							3.55	40	.81	7.38	37	22.2
After Diamox	150							3.62	38	.80	7.37	39	22.0

* Collected under oil.

TABLE II. Effects of Diamox (6063) on Man during Mild Exercise.

	Time, min.	V, ml/min.	Urine Bicarbonate, mM/L	$U_{Na}V$, μEq/min.	U_KV, μEq/min.	pH	C_{CR}	Respiratory gases V_E, M^2/BSA	V_{O_2}, M^2/BSA	RQ	Arterial blood pH	Pa CO_2, mm Hg	Total CO_2, mM/L
Rest	0	.7		50		6.81	133	4.98	141	.89	7.41	42	23.4
Exercise	45	3.3	4.35	51	41.4	6.73	140	6.9	267	.82	7.41	42	23.0
Diamox	54												
Rest	75	5.77		165	180	7.3	156	4.33	146	.80	7.39	42	20.3
	110	7.22		446	380	7.7	148						
	125	4.34	54.8	335	293	7.7	126						
Exercise	147	3.64	55.2	396	298	7.8	138	6.68	253	.81	7.36	44	21.8
	212	2.19		314	234	8.0	123						

tients were trained by repeated cardio-pulmonary studies until they could relax with constant oxygen consumption and RQ despite arterial puncture and gas collection. Two of the subjects were studied at rest, the other was also studied during a steady state of mild, active leg exercise.

Methods. After a period of rest an indwelling urethral catheter and brachial arterial needle were placed in position. Observations were begun ½ hour later. Expiratory gas, arterial blood and urine were collected and analyzed according to methods described elsewhere(7). End expiratory gas was collected by a Rahn(8) sampler and analyzed for CO_2 and O_2 using the micro-Sholander gas apparatus. Blood and urine CO_2-contents were determined by the method of Van Slyke and Neill(9). Arterial blood pCO_2 was calculated from the CO_2 content, pH and per cent oxygen saturation, using the line charts of Van Slyke and Sendroy(10), pH was determined directly by the McInnes-Belchen glass electrode. Potassium and sodium concentrations were determined by an internal standard flame photometer. Glomerular filtration rate was estimated from the endogenous creatinine clearance (C_{CR}) using the method of Bonsnes and Taussky(11). After 2 control periods, 50 mg per kg body weight of Diamox‡ were administered orally. This dose is approximately 5 times that needed to produce an alkaline urine in man, as reported by others. Observations were then repeated one and 2 hours later.

Results. In the resting subjects, Diamox caused an increase in the excretion of sodium, potassium and bicarbonate, and an increase in urine flow. (Table I). These renal effects were not associated with any change in minute ventilation V_E, oxygen consumption V_{O_2}, respiratory exchange ratio (RQ), arterial or alveolar carbon dioxide tension (Pa_{CO_2} and PA_{CO_2}). Mild exercise, sufficient to double the oxygen consumption, did not reveal any impairment in the elimination of carbon dioxide (Table II).

These observations demonstrate that in the resting subject, Diamox may have a substantial renal effect with no interference with the passage of CO_2 from pulmonary capillary blood into alveoli. The combination of Diamox plus increased CO_2 production induced by mild exercise also failed to impede CO_2 elimination into the alveoli. This failure to influence pulmonary CO_2 excretion may be due to the large concentration of carbonic anhydrase in the human erythrocyte as compared to other tissues.

Summary. Diamox (6063) was administered per os to 3 human subjects without pulmonary disease in single doses of 50 mg per kg body weight. This dose elicited typical renal effects (diuresis and increased excretion of bicarbonate, sodium and potassium) but failed, even when combined with mild exercise, to interfere with CO_2 elimination from pulmonary capillary blood into alveolar air.

‡ Diamox was kindly supplied to us by the American Cyanamid Co., Stamford, Conn.

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Chemistry of Acid-Fastness.* (20588)

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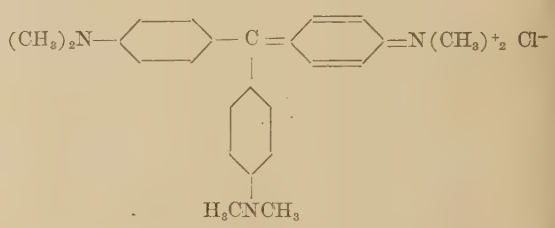
Recent work from this laboratory(1) has shown that the acid-fastness seen in mycobacteria resided in two different properties of the cells. First, there was an acid-fastness dependent upon cell structure, and hence shown by intact but not by crushed tubercle bacilli. Secondly, in the same cells there was an acid-fastness not altered by structural changes but rather dependent upon chemical composition. This second type of acid-fastness was lost only when compounds of the mycolic acid type were removed from the cells. Further, purified mycolic acid showed this same type of acid-fastness to the same degree. Lepra bacilli and sperm showed only the second type of reaction corresponding to the reactions respectively of leprosinic acid and the similar lipid recently found in human sperm(2).

The next logical step seemed to be a study of the reaction between mycolic acid and a basic dye. Contrary to previous beliefs(3) a stoichiometric relationship was found to exist between acid and dye. In addition the complex formed between mycolic acid and the dye was found to have specific and particular properties that set it off from combinations of the same dye with other organic acids.

Materials. Several samples of mycolic acid and leprosinic acids as well as the model acids tested were obtained from Dr. E. Mylon of this department. Highly purified leprosinic acid and the methyl, acetyl, and methyl-acetyl derivatives thereof were generously provided by Dr. C. A. Hargreaves, Dept. of Chemistry.

Stain-Commission certified crystal violet (C.I. 681, lot No. NC 32) was the dye chosen for the study. Although all basic dyes give the acid-fast reaction(4), crystal violet had several advantages. It was the only dye, brilliant in the visible range, which was completely removed from non-acid-fast materials by the standard acid-alcohols. Most other dyes left some residual stain in all non-acid-fast tissues, and the fuchsins left a large amount. This absence of non-specific staining offered the hope that there might also be fewer complicating chemical reactions encountered. Further the dye had a symmetrically resonating structure:



which it was thought might simplify the interpretations of the results.

Results. A. *Determination of dye-acid equilibria.* The method, adapted from Glasstone(5), was based on the fact that while the dye was soluble both in water and chloroform, the mycolic and leprosinic acids were soluble only in the latter solution.

Approximately 10 mg of dye was accurately weighed and dissolved in 500 ml of water. 100 ml of this solution was mixed with 25 ml of chloroform. In a second flask, 150 ml of the aqueous dye was added to 25 ml of chloroform containing about 10 mg of the acid in question. The movement of the dye from water to chloroform was determined colorimetrically for both systems. Equilibrium was reached for the partition of dye between water and chloroform in 6 days at room temperature (27 ± 2°C) and in 10 days for the acid-dye systems.

In the calculations it was assumed that the distribution of free dye in the two solutions was constant over the range of dye concentration studied, and that the amount of free dye in the chloroform layer was dependent on the concentration of dye in the aqueous layer

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TABLE I. Values at Equilibria for the Reaction between Acid-Fast Lipids and Crystal Violet.

Acid	mg acid/ mg dye*	g acid/ mole of dye	Molecular wt acid and source
Mycolic	3.20	1300 ± 100	
	3.16	1290 ± 100	1284 (6)
Leprosinic	2.97	1210 ± 100	{1250 / 1350} (7)

* Dye wt corrected to a purity of 91% (by titration) as given by the certification analysis. Dye molecular wt 408.

but not on the amount of dye combined with the acid. If these assumptions were true, since the amounts of dye in the aqueous layers at the beginning and the end of the experiment were known, the amount of dye in the chloroform layer could be calculated. That in excess of the dye in equilibrium with the water would be combined with the mycolic or leprosinic acid. The results of the experiment are given in Table I. It is seen that the amount of fatty acid equivalent to one mole of dye was approximately equal to the molecular weight of the acid. This ratio was *independent* of the amount of excess dye present. Hence, within the limits of the technic the reaction may be considered to have gone to completion. Most striking perhaps was the fact that the 1:1 ratio held between dye and leprosinic acid although that acid is known to possess 2 carboxyl groups. This suggests that the 2 acid radicals are not equivalent. The difference may lie in the other reacting groups near the active carboxyl (see below). In any case, the reaction appeared different enough to merit further investigation of the complex which was formed.

B. *Characterization of combination between crystal violet and acid-fast lipids.* The complex formed between dye and mycolic or leprosinic acid was soluble as were the acids themselves although the dye alone was not. When one of these acids was added to xylene layered over the dry dye, the complex was rapidly formed as evidenced by the appearance of intense color in the solution. Excess dye was then removed by filtration. Xylene was removed by evaporation. The material was redissolved in chloroform, the common solvent for the spectral determinations reported hereafter. The visible and ultra-violet spectra of the complexes, the pure acids, and the dye were determined with a Cary recording spectrometer. The results are shown in Fig. 1. The complexes showed the same absorption peak at 5900 R. as did the pure dye. In addition there was even more absorption in a band centered about 3500 Å which did not correspond to any similar band in the spectrum of the pure dye. Neither did mycolic or leprosinic acid absorb at or near this point. The specificity of this new adsorption band was emphasized in the study of reactions of other fatty acids. Cerotic acid, chaulmoogric acid, hexacosanoic acid, 10-methyl-hexacosanoic acid, 10-methyl docosanoic acid, cetyl benzylacetic acid, methyl behenic acid, α methyl-arachidic acid, α methyl tetracosanoic acid, α methyl stearic acid, α methyl cerotic acid, and 10 methyl tetracosanoic acid were all examined and found to be non-acid-fast. Decylmalonic acid, cetylmalonic acid, and hydnocaprylmalonic acid appeared acid-fast and formed a xylene-soluble complex with crystal violet. However, these complexes showed the same absorption curve in the U-V range as did the pure dye, with no evidence of absorption at 3500 Å.

C. *Nature of groups involved in the dye-leprosinic acid complex.* It was recently reported(8) that the carboxyl group was necessary for the reaction between dye and mycolic acid but that the hydroxyl group on the acid could be blocked without affecting acid-fastness. Since the only test of the reactivity had been staining itself, the problem was reexamined to determine whether blocking of either carboxyl or hydroxyl groups would affect the solubility and/or absorption of the

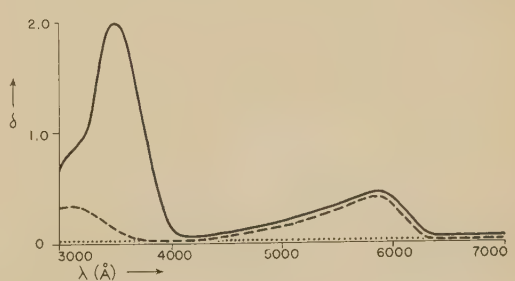

FIG. 1. Absorption spectra (in chloroform). ———— Crystal violet and mycolic acid, crystal violet and leptosinic acid. – – – – – Crystal violet, crystal violet-decylmalonic acid, crystal violet-hexacosanoic acid. ..... Mycolic acid, leprosinic acid.

product. The purified leprosinic acid gave the reactions described above. When the 2 carboxyl groups were blocked by methylation, the substance no longer formed a xylene-soluble complex with the dye, nor did a mixture of dye and ester in chloroform show absorption at 3500 Å. More surprising, exactly the same thing occurred with the compound when one hydroxyl group had been blocked by acetylation although both carboxyl groups were free. Therefore, both the acid and the hydroxyl groups appeared necessary for the *definitive* dye-leprosinic acid reaction.

Discussion. The constant stoichiometric combination of dye and acid-fast lipid, the altered solubility of the complex, and the appearance of the completely new absorption band all speak not only for the uniqueness of the reaction but for the stability of the compound which is formed. It is this stability which is described by the term "acid-fastness" as it refers to the relative difficulty with which the complex is separated by acid hydrolysis. Especially if the new absorption peak is interpreted as signifying a new resonating system in the complex, the union between mycolic acid and dye would appear far different from simple ionic relation ordinarily postulated for basic dye and acid substrate. Whether or not the specificity of the reaction lies wholly in the juxtaposition of carboxyl and hydroxyl groups, both appear necessary for the reaction and so must be considered in the formulation of any theoretical resonating structure. The present information appears insufficient however to describe the complete structure of the dye-mycolic acid complex.

Summary and conclusions. A study was made of the reaction between crystal violet and lipids of the mycolic acid type, considering this to be a prototype of the more general reaction between basic dye and acid-fast lipid. The reaction went to completion in chloroform and at the end 1 mole of dye had combined with 1 mole of mycolic or leprosinic acid. The complex so formed was soluble in xylene as well as chloroform although the dye had been soluble only in the latter solvent. The complex exhibited an intense absorption band in the near ultra violet (3500 Å) which was not present with either the pure dye or pure acids. Other acids were tested and while they might react with the dye, the specific adsorption peak was not produced. If either the carboxyl groups or the hydroxyl group of leprosinic acid were blocked the characteristic reaction did not occur.

This work was done in the laboratory of Dr. H. Bunting. In addition to Dr. Bunting, special thanks are to due to Dr. C. A. Hargreaves and J. F. Riley for their assistance and advice.

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Action of Aconitine on the Cold-Blooded Heart.* (20589)

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Early studies on the cardiac action of aconite dealt largely with its use as a "sedative". A vagal action, supposedly central and antagonized by atropine, and a direct muscle action were recognized. Renewed interest has been concerned with those abnormal rhythms that may occur spontaneously or be induced by a single properly timed electric shock in a heart under the influence of that drug. Cushny (1) early held that the arrhythmias he observed resulted from 2 factors: 1) retarded recuperation of contractile and conductive power, and 2) an increased tendency to spontaneous movements.

The purpose of the present study has been to reexamine the action of aconitine on the primary properties of heart muscle. The heart of the turtle *Pseudemys elegans* was used as isolated whole heart, isolated auricles and ventricular strips, and a few observations were made on isolated frog hearts. Preparations were immersed in oxygenated phosphate buffered Ringer solution and temperature maintained at approximately 16°C. To follow mechanical activity whole auricles or ventricular strips were suspended in a muscle bath and the beat recorded on a smoked drum. Electrical activity was recorded by a Grass 2 channel amplifier with ink writer. Aconitine nitrate in concentrations ranging from 1:1 million to 1:250 was added to the bath.

Contractility. The auricular beat was early reduced by vagal action and could be promptly restored by atropine. This negative inotropic effect was independent of rate change. Later, contraction decreased either because of rate increase or direct muscle action. Contractility of ventricular strips was usually depressed, though at times the amplitude was maintained or temporarily increased, in spite of a higher rate of beating. Alternation, an effect emphasized by Cushny, rarely appeared in these preparations. Late muscle relaxation was the rule, and even with high concentrations diastolic shortening, a non-specific toxic effect, rarely occurred.

Rhythmicity. The rate response of the spontaneously beating auricle was variable and not always dependent on drug concentration. Usually, slowing and often standstill occurred within 2 or 3 minutes, or up to 12 minutes after adding the drug. From 10 to 20 minutes after that effect progressive quickening began and a high rate was maintained. The maximum rate recorded was 90 per minute. Early slowing or standstill was promptly relieved by atropine. In a few auricles early and progressive quickening occurred without previous slowing. Both early and late quickening could be slowed by acetylcholine, at times to standstill. In the initially atropinized auricle (atropine 1:10,000) brief slowing sometimes followed aconitine, but rate increase soon occurred. Extrasystoles were infrequent. In one frog preparation but in none of the turtle auricles did spontaneous fibrillation appear. In 2 spontaneously beating ventricular strips rate increase began 15 minutes after aconitine was added; in a third slowing with periods of standstill appeared after 3 minutes, with recovery 40 minutes later. In rhythmically stimulated ventricular strips spontaneous beating began from 3 to 90 minutes after aconitine, earlier with the higher concentrations. The maximum rate of spontaneous beating for these strips was 40. The appearance of spontaneous beating was independent of the effect of the drug on the threshold for electrical stimulation and could occur when threshold had been raised. With high concentrations extrasystoles and bigeminy appeared late and 1:500 caused late slowing. Ventricular fibrillation did not occur in any of the turtle preparations but appeared in one

* This work was supported by funds of a grant from the Division of Research Grants and Fellowships, the National Institutes of Health (National Heart Institute), U. S. Public Health Service.

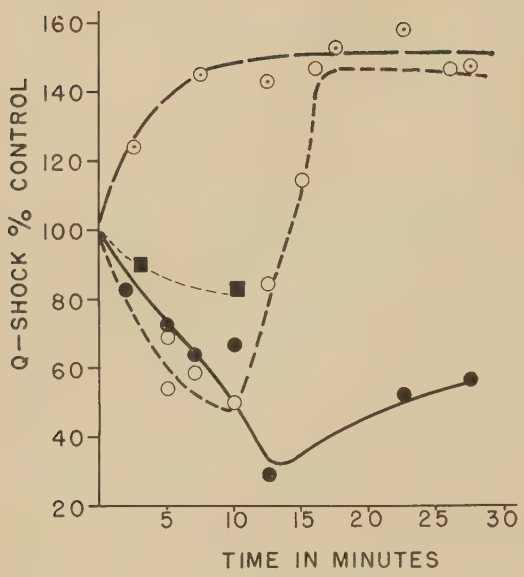

FIG. 1. Aconitine on auricular refractory period, measured as minimal shock to Q interval and expressed as % of control. Solid circles, aconitine alone (see text). Open circles, aconitine 1:50000 at zero time; atropine added after 10 min.; mean of 2 experiments. Dotted circles, aconitine 1:50000 added 12 min. after atropine at zero time; mean of 5 experiments. Solid squares, aconitine 1:500, 20 min. after atropine; 2 experiments. Atropine concentration 1:10000 in each instance.

of 5 frog hearts studied, and at a time of normal auricular activity. This sequence in the frog heart under aconitine was also observed by Cash and Dunstan(2) and suggests the importance of the vagus for the development of fibrillation. Rate increase in the spontaneously beating auricle and the initiation of spontaneous beating in ventricular preparations constitute the outstanding action of aconitine. The fact that in both preparations initial electrical wave forms were almost always maintained suggests that the effect is due to enhancement by the drug of the intrinsic rhythmic mechanism of the tissues.

Refractory period. In the spontaneously beating auricle aconitine greatly shortened the refractory period, measured as the minimal interval from a normal beat to the earliest shock followed by response. Shock strength and duration were kept constant throughout each experiment. Results for 4 groups of experiments, expressed as % of the control period, are shown in Fig. 1. Because of negligible differences with concentrations of 1:50,000 and 1:100,000 the mean value for those 2 in 8 experiments was used (solid circles). The apparent rise which began 15 minutes after the drug may be explained by threshold increase due to the drug or to increased rate of beating while shock strength remained unchanged. For drug concentrations up to 1:1000 the shortening was entirely vagal and promptly terminated by atropine (open circles). It has been shown that high concentrations of atropine affect the refractory period of the turtle heart only by vagal antagonism(3). In the initially atropinized auricle with aconitine concentrations less than 1:1000 the refractory period was not shortened. With concentrations of 1:500, shortening was produced by direct muscle action in a few experiments, but usually the early threshold rise and rate increase prevented refractory period measurement. In the forecited observations no distinction was made between single and multiple responses to a single shock. In certain auricles response followed shocks that arrived on the downstroke of the depolarization wave. The interval between that early point and the peak of the repolarization wave usually represented the time during which multiple response might follow single shocks. The period which separated single and multiple responses was highly critical, ranging from 0.05 to 0.10 sec. During

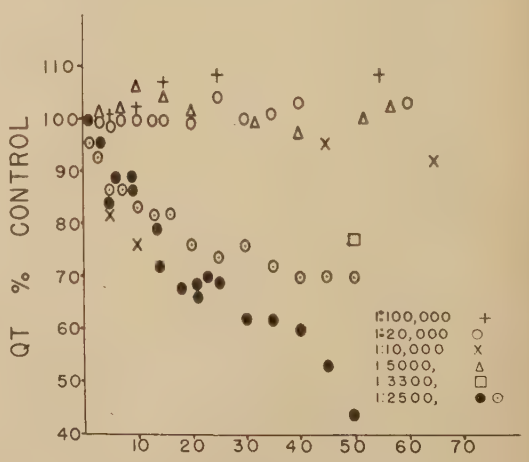

FIG. 2. Aconitine on ventricular refractory period, expressed as % change of Q-T interval of electrogram.

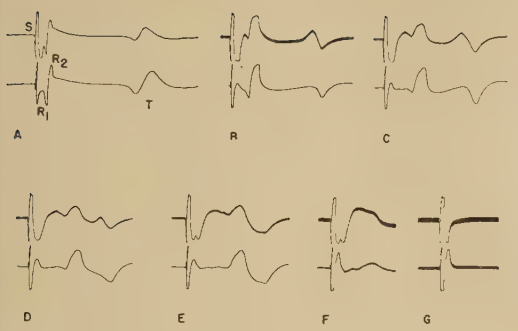

FIG. 3. Effects of aconitine 1:2500 on electrograms of a driven ventricular strip. A. Control. After aconitine: B. 11 min.; C. 25 min.; D. 40 min.; E. 45 min.; F. 50 min.; G. 60 min., no response.

auricular standstill due to aconitine the tissue responded regularly to a rapid series of shocks, and properly placed double shocks could evoke multiple response. Observations on the multiple response reaction seen in these experiments and its possible relation to the genesis of spontaneous arrhythmias will be published separately(4). Because of the tendency of aconitine to cause early spontaneous beating in driven ventricular strips, examination of its effect on ventricular refractory period was limited to a few observations on the Q-T interval of the electrogram. Although response to shocks arriving before T waves in the aconitine treated auricle suggests that the Q-T interval may not always be equivalent to the absolute refractory period, as was found in the normal and digoxin treated turtle ventricle(5), they may be expected to vary in the same direction. Only with concentrations greater than 1:5000 did significant Q-T shortening occur, Fig. 2. Marked Q-T shortening by 1:2500, followed by the appearance of diphasic and finally monophasic complexes is illustrated in Fig. 3. Such conversion to monophasic complexes by aconitine was early noted in the frog heart by Hermanns(6). Multiple response was not obtained in ventricular strips.

Threshold and conduction. The effect on threshold for electrical stimulation was observed in the ventricular strips from which the beat was mechanically recorded. Again, the early appearance of spontaneous beating limited the study. With aconitine concentrations up to 1:32,000 either slight increases or decreases occurred but in only one of 12 experiments was there a significant and sustained rise, Fig. 4. Rate increase alone will raise the threshold for stimulation. With a concentration of 1:500 the threshold for single extra shocks placed at the end of the recovery period rose promptly and even doubled before the

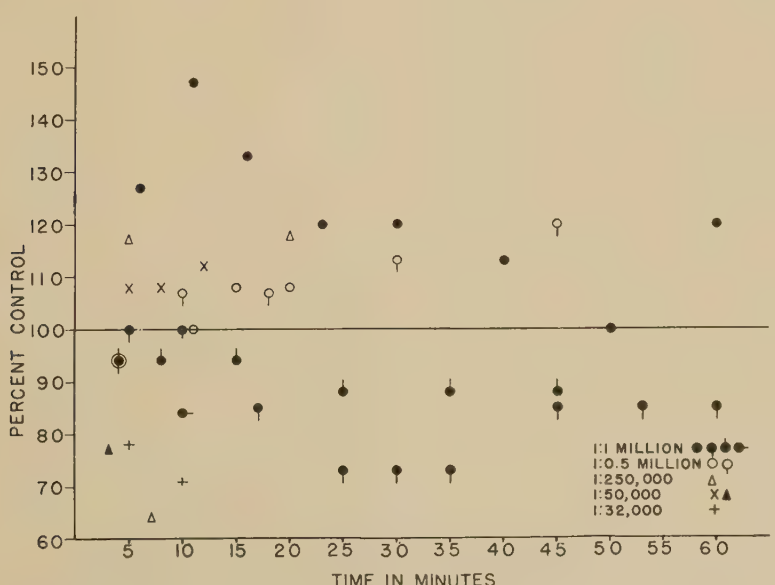

FIG. 4. Aconitine on resting threshold for electrical stimulation of turtle ventricle, expressed as % of control.

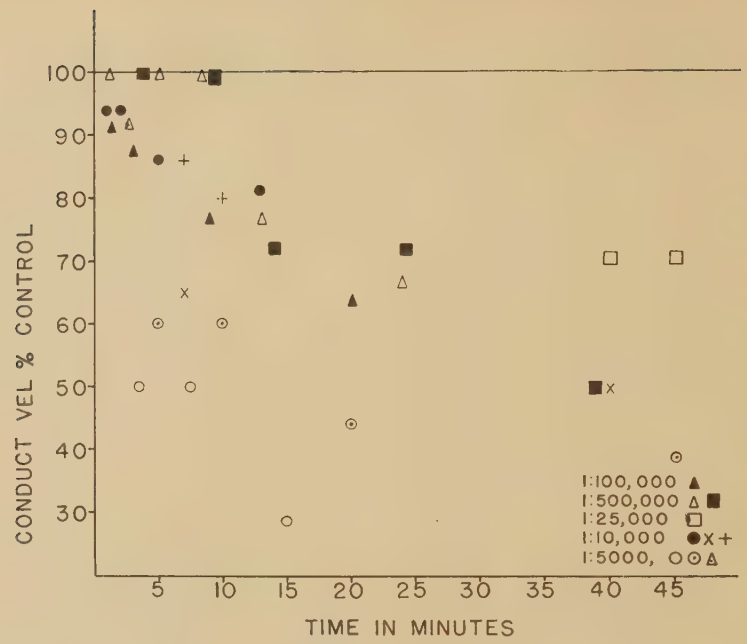

FIG. 5. Aconitine on conduction velocity in strips of turtle ventricle driven at 15 beats/min., expressed as % of control.

rate rise due to spontaneous beating. Subsequent threshold rise exceeded that anticipated for rate increase. Resting excitability was further studied in 5 preparations by determining strength-duration curves for threshold rectangular shocks that would drive ventricular strips at 15 per minute. With aconitine concentrations of 1:25,000 and lower minor variations in the rheobase were seen but calculation of chronaxie revealed no significant change. The early onset of spontaneous beating prevented detailed excitability studies with higher concentrations. As far as could be determined the appearance of a spontaneous rhythm was independent of any excitability change. *Conduction velocity* in driven ventricular strips was estimated from the R-R peaks of the electrograms with unipolar electrodes separated by about 15 mm. For the experiments recorded in Fig. 5 decreased velocity is shown. It is recognized that these may represent selected experiments since in others the distal intrinsic R deflection could not be identified. Moreover, in tissues with relatively poor initial conduction further depression will be more readily produced. There seemed no doubt, however, that aconitine was able to depress conductivity, at times by comparatively low concentrations.

In driven ventricular strips the electrical latent period, the interval from the shock to the depolarization wave, was prolonged only by aconitine concentrations greater than 1:20,000 (Fig. 3).

The QRS complex of certain ventricular electrograms was appreciably prolonged by aconitine, an effect not always related to concentration. In Fig. 3, showing the response to 1:2500, QRS after 60 minutes was 120% of the control. In another experiment the QRS interval was 150% of the control 30 minutes after 1:100,000. In many experiments the magnitude of the complexes was increased. The reason for those changes is not apparent. Since the electrodes were small wicks and the leads practically unipolar, such alteration may not be due solely to conduction effects but may represent some slowing by the drug of the depolarization process itself.

Summary. The effect of aconitine on the primary properties of heart muscle has been studied in the cold-blooded heart. Its constant action was to increase the rate of spontaneous beating or to initiate a spontaneous

rhythm in driven ventricular strips. This action appeared to be independent of change in resting excitability and to result from enhancement of intrinsic rhythmicity. Vagal action on the auricle depressed the pacemaker, decreased contraction amplitude and shortened refractory period. Contractility and refractory period changes were independent of rate change. In the atropinized auricle high concentrations shortened refractory period by direct muscle action. During the stage of shortened refractory period there was a critical interval when a single extra shock might elicit multiple responses. In the turtle ventricle, which has no vagal endings, high concentrations shortened refractory period, decreased conduction velocity and raised threshold for electrical stimulation.

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Effects of N-Methylformamide and Related Compounds in Mouse Sarcoma 180.* (20590)

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Recently a series of formamides was evaluated as possible solvents for parenteral administration of organic chemicals in the sarcoma 180 screening program of this Institute(1). The compounds proved too toxic for this purpose. However, 2 of the agents, formamide and N-methylformamide (Fig. 1), were found to inhibit the growth of the assay tumor. A third, N,N-diethylformamide, had questionable inhibitory activity whereas other members of the series were without effect. The present report is a study of the inhibitory actions of the formamides in sarcoma 180 (S-180). Special emphasis is given to the N-methyl derivative since it appears to be the most effective of the series. The inhibitory action of urethane against S-180, which has been mentioned previously(1), is also reviewed since the chemical configuration of this agent resembles that of the formamides (Fig. 1) and since it has been found effective against a variety of experimental and clinical neoplasms(2-5).

Tumor inhibition test. Female mice of the Swiss-Webster (CFW) strain weighing 18-22 g were employed. Small, uniform cubes (*ca*

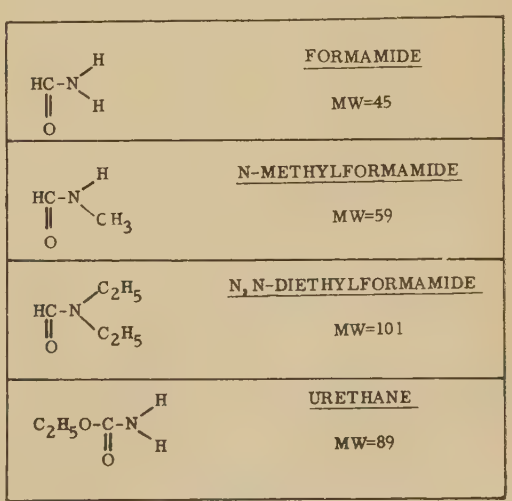

FIG. 1.

* This study was supported in part by an institutional grant from the American Cancer Society and in part by funds from the National Cancer Institute, U. S. Public Health Service.

TABLE I. Inhibition of Growth of Mouse Sarcoma 180 by Formamides. Treatment initiated 24 hours after implantation.

Compound	Mean diameter of tumors, mm ± SD	Toxicity: Avg wt change, g‡	Toxicity: Mortality
I. Observations at end of therapy			
N-Methylformamide*	3.1 ± 1.0	—4.5	1/30
Controls	11.9 ± 1.7	— .5	0/30
Formamide*	6.4 ± 1.6	—4.0	0/30
Controls	11.7 ± 1.2	—1.0	0/30
Diethylformamide*	10.3 ± 1.5	+ .5	0/30
Controls	11.2 ± 1.3	+ .5	0/30
Urethane†	6.7 ± 1.1	—4.0	3/30
Controls	11.4 ± 1.5	— .5	0/30
II. Observations 1 wk after end of therapy			
N-Methylformamide	10.2 ± 1.9	—2.0	9/30
Controls	14.4 ± 2.0	—5.5	7/30
Formamide	11.3 ± 1.0	—4.4	11/30
Controls	13.8 ± 1.1	—5.5	6/30
Diethylformamide	14.5 ± 2.5	—4.0	2/30
Controls	14.0 ± 2.9	—3.5	3/30
Urethane	10.6 ± 1.8	—6.5	18/30
Controls	15.3 ± 2.0	—5.0	4/30

* Given intraperitoneally, 500 mg/kg/day.
† " " ,1000 "
‡ Cumulative from day of implantation.

2 x 2 x 2 mm) of mouse S-180 were implanted by trocar subcutaneously in the right axillary region of recipient mice. Treatment was initiated either 24 or 96 hours later. The agents† were dissolved in isotonic NaCl and administered either by intraperitoneal injection or by oral intubation in the volume of 0.5 ml/20 g mouse once daily on each of 7 successive days. Control animals were simultaneously injected with a similar volume of isotonic NaCl. One day after completion of therapy tumor diameters in two perpendicular axes were measured through the skin by means of vernier calipers; the averages of the two diameters were used to obtain the mean diameter of each experimental group.

Results and discussion. Treatment initiated 24 hours after implantation. Part I of Table I illustrates that significant inhibition of tumor growth was obtained by the end of therapy in those animals treated with approximately equimolar doses of N-methylformamide, formamide, and urethane. Since the weight of S-180 varies directly as the cube of its mean diameters(6), it was possible to calculate that the ratio of tumor mass in animals given N-methylformamide to that in controls was 1:57; in those given formamide and urethane the proportions were 1:6.1 and 1:4.9, respectively. On the other hand, the effect of N,N-diethylformamide was relatively negligible; the ratio of mass in treated and control tumors was 1:1.3. Moreover, N,N-diethylformamide failed to cause significant inhibition of S-180 when given in the dose of 1120 mg/kg/day which is equimolar to the dose of formamide employed in Table I. It thus appears that the N-methyl group enhances the activity of the formamide moiety while other substitution suppresses inhibitory effects.‡

The results in Table I also indicate that the inhibitory effects noted were not persistent. Part II of the table describes the status of tumors in the animals which survived for as long as one week after the end of therapy. By this time the treated tumors had resumed rapid growth and the inhibitions noted one week earlier were now less evident. A further analysis of the recovery of S-180 from inhibition by N-methylformamide will be presented below. The fatalities, recorded in Part II, Table I, in groups treated with the 3 active substances were presumably due to intoxication by the agents, for the majority of these deaths occurred during the week of treatment or within the first 48 hours thereafter. Moreover, the size of tumors in these animals at time of death was significantly less than that found in untreated, tumor-bearing mice(6). On the other hand, in the control groups and in mice given N,N-diethylformamide, fatalities occurred in animals bearing tumors of a size usually associated with death due to intoxication by S-180(6). The dose of urethane employed was sufficiently depressant to mice to result in loss of righting re-

† The authors are indebted to the Rohm and Haas Co., Philadelphia, for generous supplies of purified N-methylformamide.

‡ Preliminary observations with more than 60 different formamides have failed to uncover any which are inhibitory to S-180 other than formamide and its N-methyl derivative. Of particular interest has been the finding that acetamide, N,N-dimethylformamide, N-ethylformamide, and thioformamide were among the ineffective formamides.

TABLE II. N-Methylformamide Inhibition of S-180 Growth in Mice. Intraperitoneal *vs.* oral routes. Treatment initiated 24 hours after implantation.

Dose, mg/kg/day	Intraperitoneal route: No. of mice surviving	Intraperitoneal route: Mean diameter of tumors, mm ± SD	Oral route: No. of mice surviving*	Oral route: Mean diameter of tumors, mm ± SD
500	10/10	2.6 ± .5	10/10	2.5 ± .5
400	"	2.6 ± .6	"	2.9 ± 1.4
300	"	3.1 ± 1.2	"	4.1 ± 1.4
200	"	6.1 ± .9	9/10	6.1 ± 1.2
100	"	8.3 ± 1.7	8/10	8.1 ± 1.4
Controls	20/20	10.8 ± 1.2	11/20	10.5 ± 1.2

* Deaths were all accidental, incident to intubation.

flexes and to cause complete immobilization for several hours after each injection. It is likely that the high incidence of mortality among urethane-treated animals was, in part, attributable to central depression. On the other hand, no depressant actions were observed after administration of either formamide or its two derivatives. It will be noted in Table I that the active agents caused marked weight loss by the end of therapy. This debilitation cannot be considered the sole cause of tumor inhibition for the growth of S-180 is only moderately affected in mice which have been even more severely debilitated by dietary restriction. As an example, mice have been placed on a regimen of 0.5 g of diet§/individual/day with water *ad lib* at 24 hours after tumor implantation. They lost an average of 6 g in body weight over a period of 8 days while their tumors grew to a mean diameter of 8.0 mm. Control mice receiving both food and water *ad lib* during this period gained an average of 0.5 g in body weight and the mean diameter of their tumors was 10.9 mm(6). Furthermore, numerous examples have recently been reported of compounds which cause marked weight loss without significant inhibition of S-180(7). The response of the growth of S-180 to varying doses of N-methylformamide is shown in Table II. The table also indicates that the agent is equally effective whether given by intraperitoneal injection or by oral intubation.

TABLE III. N-Methylformamide Inhibition of S-180 Growth in Mice. Treatment initiated 96 hours after implantation.

Treatment	No. of mice	Mean diam. of tumors (mm ± SD) at Start of therapy	Mean diam. of tumors (mm ± SD) at End of therapy
N-Methylformamide*	15	5.4 ± .7	4.1 ± .7
Controls	15	5.0 ± .9	14.1 ± 1.9

* Intraperitoneal injections, 500 mg/kg daily for 7 days.

§ Purina Laboratory Chow.

Treatment initiated 96 hours after implantation. As in the case of 24-hour implants, the growth of 96-hour tumors was inhibited by N-methylformamide (Table III). This finding is to be contrasted with the effects of other agents previously shown to be active against S-180. For example, 2,4,6-triethylenimino-*s*-triazine(8) and certain of the folic acid antagonists(9) produce marked inhibition when therapy is initiated in mice with 24-hour implants; however, these agents are relatively ineffective when therapy is delayed until 96 hours. This difference between N-methylformamide and the last mentioned agents cannot be explained at present. It may, however, be related to changes in metabolism of S-180 between 24 and 96 hours after implantation, for implants require at least 48 to 72 hours before they become vascularized and attain maximal rates of growth(6). The transient nature of the inhibition caused by N-methylformamide has been noted above in the results shown in Table I. As in the previous experiment, the treated tumors of Table III resumed growth during the week following the end of therapy. In 3 other experiments mice with 96-hour implants were given a single injection of N-methylformamide which caused transient inhibition, as illustrated in Fig. 2, without inducing significant loss of body weight.

Microscopic alterations in treated tumors. The microscopic effects of N-methylforma-

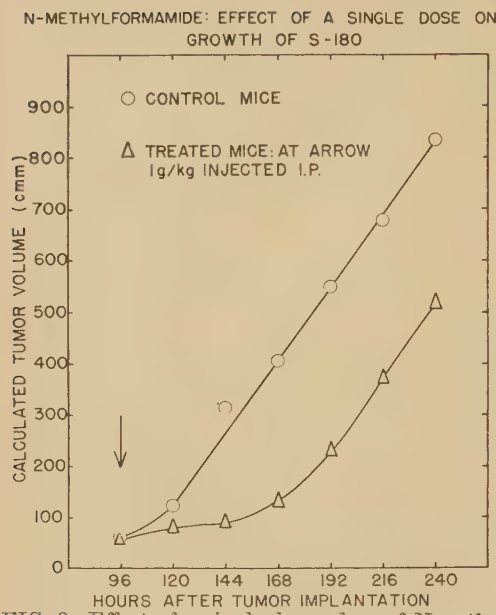

FIG. 2. Effect of a single, large dose of N-methylformamide on growth of S-180. Curves are avg tumor volumes of 15 controls and 15 treated mice; volume of each tumor calculated from its mean diameter.

mide were studied in tumors from mice given successive daily injections of 500 mg/kg/day (treatment initiated with 24-hour implants). Groups of 3 animals were sacrificed at 24-hour intervals from 1 to 8 days after initiation of therapy. Earliest evidence of microscopic alteration was found in tumors removed at 3 days; thereafter, cellular changes became progressively more pronounced. Briefly, the aberrations included the presence of enlarged cells with swollen, frequently vacuolated cytoplasm and with abnormal nuclei exhibiting either bizarre distributions of chromatin or pyknosis. Although such alterations represent significant changes in tumor cells, their non-specific nature deserves emphasis, for similar disturbances have been found in S-180 treated with certain other inhibitory agents (*e.g.*, folic acid antagonists(10), 2,4,6-triethylenimino-*s*-triazine(11) and N, N′, N″-triethylenephosphoramide(12)).

Mode of action. Formamide and N-methylformamide, like amides in general, are relatively stable substances. Therefore the possibility of direct chemical combinations with vital cellular constituents seems unlikely unless such reactions were to involve unknown enzymatic processes. In regard to the latter it should be kept in mind that the products of the hydrolysis of the agents, namely, formic acid, ammonia, and methylamine, are biologically active substances. It is also conceivable that formamide and its N-methyl congener could act as metabolite antagonists for certain simple substances which closely resemble the agents in molecular configuration. Specifically, it is tempting to propose an antagonism of single carbon units, for the role of one-carbon intermediates in vital biosynthetic mechanisms is well-established(13-15). In a preliminary test of this hypothesis the administration of formate, acetate, and glycine failed to block the inhibitory effects of the formamides in S-180 or to reduce their toxicity.‖ Since antagonists of folic acid are known to interfere with incorporation of formate into polynucleotide adenine, guanine, and thymine(16, 17) and since such antifolics are known to be potent inhibitors of S-180(9), attempts were made to prevent formamide inhibition with either folic acid or synthetic citrovorum factor. These two metabolites also failed to block tumor inhibition or toxicity.‖

Another possible mechanism was suggested by the observation of Skipper, *et al.*, that both urethane and formamide inhibit the growth of *Escherichia coli.* Inhibition of growth by either agent was prevented by the presence of small amounts of 2,6-diaminopurine (DAP) or of its riboside(18,19). DAP was tested as a possible antagonist of the formamides in S-180 but in the manner employed failed to influence the tumor inhibition

‖ All blocking experiments were started 24 hours after S-180 implantation. In separate experiments, formamide and N-methylformamide were injected in doses of 500 mg/kg/day for 7 consecutive days. The dose of the candidate blocking agent was injected just prior to the dose of the inhibitor. The following daily doses of these candidates were employed (mg/kg): sodium formate, 500; sodium acetate, 500; glycine, 500; folic acid, 25; synthetic citrovorum factor (leucovorin), 10; 2, 6-diaminopurine, 35. The authors are grateful to Dr. J. M. Ruegsegger of the Lederle Laboratories Division of American Cyanamid Co., Pearl River, N. Y., for supplies of leucovorin and to Dr. G. H. Hitchings, The Wellcome Research Laboratories, Tuckahoe, N. Y., for supplies of 2,6-diaminopurine.

or toxicity caused by the latter substances.‖

Finally another possible mode of action of the formamides is suggested by their hepatotoxicity, a property which will be the subject of a future publication. The toxic actions of both formamides in mice, rats, and dogs are referable to hepatic dysfunction and, following large doses, parenchymal necrosis. Moreover, preliminary studies have revealed that mice, in which the growth of S-180 has been inhibited, also evidence a simultaneous hepatic insufficiency. Whether or not tumor inhibition in mice reflects indirectly a primary hepatic damage is at present under investigation. It is of some interest to note that urethane itself has been found hepatotoxic(20, 21) although a possible relationship between this action and its capacity to affect experimental and clinical tumors has not previously been considered(2-5).

Summary and conclusions. 1. Formamide and its more potent N-methyl derivative have been described as transient inhibitors of the growth of mouse sarcoma 180. This effect is not a property of formamides in general since other compounds containing the formamide moiety have failed to affect the growth of the tumor. 2. N-methylformamide has been shown to exert its effects when therapy is started either 24 or 96 hours after implantation of the tumor or when the agent is given in a single, large dose. Further, the compound has been found equally effective whether given by the oral or intraperitoneal route. 3. Histologic changes of a non-specific nature have been described in tumors from animals treated with N-methylformamide. 4. In view of the structural resemblance between formamide and urethane, the actions of both agents have been compared. Urethane, like formamide, inhibits the growth of S-180; however, treatment with the former substance causes toxic manifestations not encountered in mice given formamide. 5. Several conceivable mechanisms have been proposed to account for the inhibitory effects of the formamides. Further studies of these suggested modes of action may provide useful information concerning biochemical mechanisms involved in the growth of tumors. 6. In view of the hepatotoxicity of formamide and its N-methyl derivative it is not expected that these agents will prove therapeutically useful.

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Excretion of N^1-Methylnicotinamide and the 6-Pyridone of N^1-Methylnicotinamide in Urine of Human Subjects.* (20591)

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The major excretory metabolites of nicotinamide in human subjects are N^1-Methylnicotinamide (N^1-Me) and the 6-pyridone of N^1-Methylnicotinamide (pyridone)(1-4). After a test dose of nicotinamide, pyridone is excreted in larger quantity than N^1-Me and in persons receiving diets low in niacin and its precursors, pyridone excretion decreases more rapidly than N^1-Me. Subjects with pellagra, experimentally induced by diets low in niacin and tryptophan, excrete no detectable pyridone or only a trace of this metabolite, but continue to excrete a small amount of N^1-Me. Thus, pyridone appears to be the more important end product in nicotinamide metabolism(5). Holman and deLange(6) have studied the rate of N^1-Me and pyridone excretion during 24 hours following the ingestion of nicotinamide. The major increase in N^1-Me excretion occurred within the first 3-hour period and values fell to control levels after 9 hours. The rate of excretion of pyridone, however, was maximum during the second 3-hour period and decreased slowly toward the control level during the remainder of the 24 hours. There is a paucity of information concerning the quantitative excretion of pyridone and N^1-Me in subjects maintained on relatively constant diets of known composition. It is the purpose of this report to present data on the urinary excretion of these metabolites by human subjects on such diets and following the ingestion of a test dose of nicotinamide. Diets A and B used in this study were designed to provide minimal amounts of nicotinic acid and tryptophan. Addition of tryptophan to the diets has been shown to result in increased excretion of N^1-Me(7-11) and of pyridone(5,12) in man. In the present experiments, dietary tryptophan was maintained at a constant level, and the changes in the excretion of N^1-Me and pyridone are associated with the administration of nicotinamide.

Methods and materials. Adult human subjects of both sexes between 25 and 57 years of age, who were found to be essentially free of organic disease, and 2 subjects with pellegra were used in the study. They were maintained in a metabolism ward on standardized diets high in either corn (Diet A) or wheat (Diet B) as previously described(5). Analysis of the diets showed a daily intake ranging from 4.3 to 6.0 mg nicotinamide and 180 to 230 mg of tryptophan. In one series of experiments, a standardized diet of better nutritional value (Diet C) was used which supplied approximately 10 mg of nicotinamide and 1 g of tryptophan daily. Urine samples were collected in dark bottles containing 5 ml glacial acetic acid. A portion of each sample was neutralized (pH 6.9 $\pm$ 0.1) and frozen until analyzed. Fluorometric methods for the determination of N^1-Me(13) and pyridone(14) were modified for use in this laboratory. Creatinine determinations using alkaline picrate reagent and total nitrogen by the macro Kjeldahl procedure were performed to check completeness of urine collections. When oral supplements of nicotinamide were administered, they were given with breakfast and subjects were permitted to eat at regular mealtimes.

Results. Six normal subjects were maintained on Diet A and 3 normal subjects on Diet B for 2 to 4 days in order to obtain basal excretion values prior to supplementation. Following this, a 50 mg test dose of nicotinamide was administered and urines were collected for 24 hours. In 4 of the subjects, the

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TABLE I. Excretion of N^1-Methylnicotinamide and 6-Pyridone of N^1-Methylnicotinamide Following Ingestion of 50 mg Nicotinamide by Human Subjects.

Subject	No. of determinations	Diet	N^1-Methylnicotinamide (mg/day)		6-Pyridone (mg/day)	
			Control	Nicotinamide	Control	Nicotinamide
E.	1	Corn (Diet A)	3.7	10.5	4.9	35.5
K.	3	"	2.9	8.0	7.6	25.7
T.	1	"	4.3	12.9	4.6	36.0
O.	1	"	2.2	6.6	3.3	23.4
M.	3	"	3.1	7.3	10.0	22.7
C.	3	"	1.6	5.9	2.4	27.1
D.	2	Wheat (Diet B)	1.3	8.1	1.7	23.8
P.C.	1	"	2.4	6.8	5.0	32.1
L.	1	"	4.2	9.2	3.2	25.2
		Avg ± S.E.*	2.9 ± .36	8.4 ± .73	4.7 ± .91	28.1 ± 1.74

* S.E. (stand. error) $= \sqrt{\frac{\Sigma d^2}{N\,(N-1)}}$. This formula refers to all such data in this report.

TABLE II. Excretion of N^1-Methylnicotinamide and 6-Pyridone of N^1-Methylnicotinamide During the First 4 Hours and Subsequent 20 Hours Following Ingestion of 50 mg Nicotinamide by Human Subjects.

Subjects	No. of determinations	Diet	N^1-Methylnicotinamide (mg)		6-Pyridone (mg)	
			4 hr	20 hr	4 hr	20 hr
M.	6	Corn (Diet A)	4.2	3.4	11.5	15.7
K.	5	"	4.2	2.8	10.0	16.2
H.	1	"	3.6	5.5	6.5	19.6
C.	3	"	3.8	2.1	11.1	16.0
M.M.	1	"	1.7	2.0	6.7	15.1
D.	1	"	5.2	3.7	11.6	16.5
T.	1	"	6.7	6.2	12.0	24.0
B.	1	"	5.6	4.4	8.0	19.0
O.	1	"	3.3	3.3	8.4	14.9
H.	2	"	3.0	2.9	6.2	11.8
V.D.	2	Wheat (Diet B)	4.3	3.5	6.6	9.0
P.C.	1	"	3.2	3.5	13.4	18.7
		Avg ± S.E.	4.1 ± .37	3.6 ± .37	9.3 ± .71	16.4 ± 1.10

tests were carried out 2 or 3 times. The values obtained in these repeated tests agreed well and were averaged to obtain the values for the individual subjects (Table I). Average excretion on the basal diet was 2.9 mg of N^1-Me and 4.7 mg of pyridone per day. Following administration of a 50 mg test dose of nicotinamide there was a 4 to 8 mg increase in the excretion of N^1-Me (average 5.5 mg) and a 13 to 31 mg increase in the excretion of pyridone (average 23.4 mg) above basal levels. When calculated on an equivalent basis, the average excretion of N^1-Me represented 10%, and of pyridone, 39% of the nicotinamide test dose, during the 24-hour period.

The rate of excretion of N^1-Me and pyridone during the first 4 hours, and subsequent 20 hours of the 24-hour period following a 50 mg test dose of nicotinamide, was determined in 12 subjects maintained on Diets A and B (Table II). Data for 6 of these subjects were included in Table I. In some subjects more than one determination was carried out. The excretion of N^1-Me averaged 4.1 mg during the first 4 hours and 3.6 mg during the succeeding 20 hours; the excretion of pyridone was 9.3 mg during the first 4 hours and 16.4 mg during the following 20 hours. These data indicate that the administration of nicotinamide is followed by a rapid increase in the excretion of N^1-Me during the first 4 hours after the test dose. Excretion of pyridone,

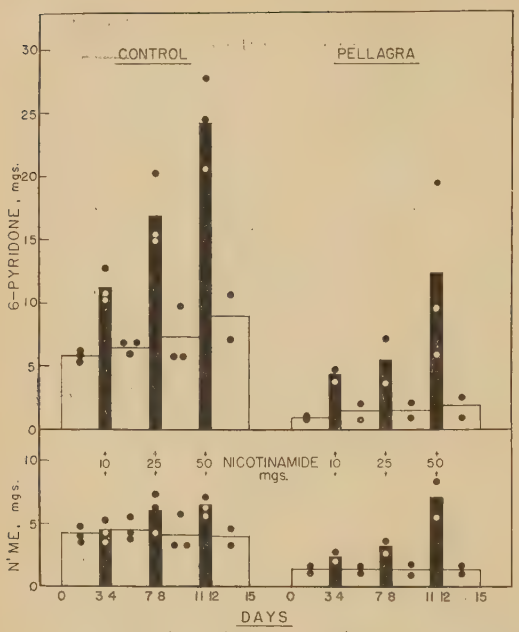

FIG. 1. Avg daily urinary excretion of N¹-Methylnicotinamide and 6-pyridone of N¹-Methylnicotinamide in 3 normal subjects and 2 subjects with pellagra on Diet C and following the administration of 10, 25 and 50 mg nicotinamide.

on the other hand, increases more slowly and smaller amounts are excreted in the initial 4 hours than in the subsequent 20 hours. The body stores of the subjects in the experiments appear to be lower than those previously found in 51 control subjects(15) who excreted 8.0 mg of N¹-Me in 4 hours following a 50 mg test dose of nicotinamide, as contrasted with 4.1 mg found in the present study. However, the excretion is similar to that found in 14 miscellaneous hospital patients who excreted 5.1 mg in 4 hours.

The excretion of N¹-Me and pyridone following graded doses of 10, 25 and 50 mg of nicotinamide was studied in 3 control female subjects and 2 female subjects with pellagra. The subjects were maintained on the basal diet (Diet C) throughout the test, allowing 3-day periods between successive test doses. This diet contained about twice as much nicotinamide and 5 times as much tryptophan as did Diets A or B. After each test dose, the 24-hour urine sample was collected and analyzed. The daily basal excretion of the control subjects on Diet C averaged 4.2 mg N¹-Me and 5.9 mg pyridone (Fig. 1). These basal values are significantly greater than those found for nine subjects on Diets A or B, who excreted 2.9 mg of N¹-Me and 4.7 mg of pyridone, daily (Table I). The subjects with pellagra, although maintained on Diet C, excreted only 1.3 mg of N¹-Me and 0.9 mg of pyridone. This excretion is much less than that of control subjects on any of the basal diets which were very low in nicotinamide and tryptophan.

The average excretion of N¹-Me by the control subjects following a 10 mg test dose of nicotinamide increased only slightly above the basal level, but that of pyridone increased by almost 5 mg (Fig. 1). Following doses of 25 and 50 mg, the excretion of both metabolites increased significantly above basal levels. The pyridone excretion, however, increased more than did N¹-Me. Subjects with pellagra excreted considerably less N¹-Me and pyridone following a 10 or 25 mg dose of nicotinamide than did control subjects. Following a 50 mg dose, excretion of N¹-Me was similar in the 2 groups while excretion of pyridone was lower in one pellagrin, and essentially the same as the control subjects in the other. In the latter subject, pellagra was relatively mild. These findings suggest that the 50 mg dose of nicotinamide may be too large to be useful in detecting borderline cases of niacin deficiency. Determinations of N¹-Me and pyridone excretion on standard diets and following a 10 or 25 mg dose of nicotinamide, however, appear to be valuable procedures in evaluating niacin nutrition.

It may be noted that following administration of graded doses of nicotinamide, the basal excretion of pyridone increased slightly while that of N¹-Me tended to remain constant. With large doses of nicotinamide, there is probably an increase in pyridone excretion beyond the first 24-hour period, which is reflected in the values given for the 3-day basal periods.

Summary. Essentially normal subjects maintained for short periods on diets low in nicotinamide and tryptophan excrete approximately 3 mg of N¹-Me and 5 mg of pyridone per day. Following an oral 50 mg test dose of nicotinamide, the average increase in excretion of N¹-Me is 5.5 mg and that of pyridone, 23.4 mg. The excretion of N¹-Me fol-

lowing the test dose rises rapidly during the first 4 hours and decreases to basal levels within 24 hours; pyridone is excreted more slowly the first few hours but appears in large amounts later in the day. After graded doses of nicotinamide, the excretion of pyridone increases more rapidly than does that of N^1-Me. In subjects with pellagra, the excretion of these metabolites is lower both on standard diets and following administration of small doses of nicotinamide. These data suggest procedures that may be of use in evaluating niacin nutrition.

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Observations on Antibiotic Treatment of Bacterial Infections of Cortisone-Treated Mice. (20592)

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Numerous reports on the action of cortisone in promoting progression of various types of infection in man and in experimental animals have appeared in recent years, and in addition, observations on alteration of cellular response to various inflammatory stimuli have been described(1). In previous studies(2) in which *Mycobacterium tuberculosis* infection was induced in mice by the intraperitoneal route, it was observed that mice treated with large doses of isoniazid and cortisone showed much better therapeutic response than did mice treated with isoniazid alone Mice treated with large doses of cortisone and streptomycin did not give such a good response. The work was extended to gain further information on the relationship between invading bacteria and the defense mechanisms of the host; studies have been made in which experimental infections induced in mice by other bacteria were treated with appropriate antibiotics and with or without cortisone.

Methods. CF_1 male mice (18-22 g) were used in all experiments. Mice were infected by the intraperitoneal injection of cultures which had been incubated at 37°C for 24 hours. The strain of streptococcus used in most experiments (*S. zooepidemicus*—Group C) was originally isolated from a casaeus lymphnode of a guinea pig and was maintained on blood agar slants. The culture of *Streptococcus haemolyticus* (Group A) used was obtained through the courtesy of Dr. Fred B. Traub. Cultures for infecting mice were prepared in

TABLE I. Effect of Cortisone on Antibiotic Treatment of Mice Infected with Group C Streptococcus.

Antibiotic	Dosage and administration	Antibiotic + cortisone*: Dose of cortisone, mg/day	Mortality† Dead	Mortality† Total	Survival, %†	Antibiotic only*: Mortality† Dead	Mortality† Total	Survival, %†
Penicillin (procaine)	0.1 mg/ml drinking water‡	2.5§	5	38	87.	32	38	15.7
	1 " " "	.8	0	8	100.	0	8	100.
	0.25 mg/aqueous subcut. 4× daily	1.	10	10	0	10	10	0
	1 " " " 2× daily	1.	10	10	0	10	10	0
	2 " " " "	1.	10	10	0	10	10	0
	1 mg/oil " "	1.	0	10	100.	0	10	100.
	1 " " " "	.2	0	10	100.	0	10	100.
Aureomycin	1 mg/aqueous " "	2.5§	14	18	12.	0	18	100.
	2 " " " "	.8	10	10	0	0	10	100.
	2 " " " "	.2	0	10	100.	0	10	100.
Streptomycin	1 " " " "	2.5§	25	28	10.7	8	28	71.4
	2 " " " "	.8	32	40	20.	5	40	87.5
	2 " " " "	.6	5	10	50.	1	10	90.
	2 " " " "	.4	4	10	60.	2	10	80.
	2 " " " "	.2	2	20	90.	4	20	80.
Terramycin	1 " " " "	2.5§	10	10	0	3	10	70.
	2 " " " "	.8	10	10	0	1	10	90.
	2 " " " "	.2	0	10	100.	0	10	100.

* Mice treated on day 1, 2, and 3. † On the 5th day after infection. ‡ Taken *ad lib.*
§ Cortisone inj. 2.5 mg/kg on day 1, 1 mg/kg on day 2 and 3.

tryptose phosphate broth (Difco) containing 10% human serum. Laboratory stock cultures of *K. pneumoniae, P. multicida,* and *S. pullorum* were grown in tryptose phosphate broth. *Br. abortus* was grown on trypticase soy agar. Cortisone as the acetate was used in all experiments. It was injected subcutaneously as an aqueous suspension and dosage was given once a day beginning on the day of infection, and for the next two days, except in experiments with *Br. abortus* in which the mice were treated with cortisone for 5 days. The doses used in experiments, other than those with *Br. abortus,* was such that 80% to 100% of untreated mice died within 5 days of the time of infection. All of 292 infected, untreated mice, and 292 infected mice treated with cortisone only, used in experiments shown in Table I and II, died within the 5 day period of observation. Other details are described in connection with the various experiments.

Experimental. Mice infected with Group C streptococcus were treated with oral or subcutaneous penicillin with or without injections of cortisone. Procaine penicillin (1000 units/mg) was given in drinking water, by subcutaneous injection as aqueous solution twice, or 4 times a day in divided doses between 9 a.m. and 5 p.m., or by subcutaneous injection of a suspension in oil with aluminum mono-stearate further diluted in sesame oil to give the desired dose in 0.1 ml. Mice were infected between 9 and 10 a.m. by the intraperitoneal injection of 0.1 ml of serum broth culture. *Ad libitum* or subcutaneous dosage with penicillin was begun 2-3 hours after infection, and cortisone was injected within 1 hour of the time of infection. Treatment was given for 3 days, and survivors were totaled after 5 days. Groups of mice which received no treatment and groups treated with cortisone alone were included in each experiment.

It is seen in Table I that mice infected by intraperitoneal injection of Group C streptococci survive when treated with penicillin or penicillin and cortisone, provided the dosage of antibiotic is adequate. Thus, the infected mice which drank daily 1000 to 10000 units of penicillin in water or which received 1000 units in oil each day for the 3 day treatment period, usually survived, but when aqueous penicillin was injected subcutaneously 2 or 4 times during the day, but not during the night, there were no survivors. It is of interest that a greater number of mice survived among those which were given drinking water containing 100 units of penicillin per ml, and which were injected with large doses of corti-

TABLE II. Effect of Cortisone on Antibiotic Treatment of Mice Infected with Various Bacteria.

Infection	Antibiotic treatment* and dosage		Cortisone,† mg/day	Mortality‡ Dead	Mortality‡ Total	Survival, %	Antibiotic only Mortality‡ Dead	Antibiotic only Mortality‡ Total	Antibiotic only Survival, %
Streptococcus (Group A)	Penicillin 0.1 mg/ml drinking water		1.	6	10	40.	7	10	30.
		mg subcut. 2× daily							
	Neomycin	.5	1.	10	10	0	4	10	60.
P. multicida	Terramycin	1	2.5§	10	10	0	2	10	80.
S. pullorum	Streptomycin	1	2.5§	20	20	0	14	20	30.
Brucella abortus	Streptomycin	.25	.8	18	20	10.	12	20	40.
	Aureomycin	"	.8	20	20	0	5	20	75.
	Terramycin	"	.8	15	20	25.	8	20	60.
Klebsiella pneumoniae	Streptomycin	1	2.5§	16	20	20.	0	20	100.
	"	1	.8	15	20	25.	0	20	100.
	"	2	.8	0	20	100.	0	20	100.
	Aureomycin	2	.8	17	20	15.	0	20	100.
	Polymyxin B	.2	1.	8	10	20.	0	10	100.
	Neomycin	1	1.	5	10	50.	0	10	100.
	Chloramphenicol	1	2.5§	9	10	10.	0	10	100.
	Terramycin	1	.8	9	10	10.	0	10	100.
	"	"	.6	8	10	20.	0	10	100.
	"	"	.4	6	10	40.	0	10	100.
	"	"	.2	2	10	80.	0	10	100.

* Mice treated on day 1, 2, and 3.

† Aqueous suspension of cortisone acetate inj. subcut. once a day, day 1, 2, and 3.

‡ After 5 days with the exception of those infected with *Brucella abortus* which were tabulated after 14 days.

§ Cortisone inj. subcut. 2.5 mg on day 1, 1 mg on day 2 and 3.

sone (2.5, 1, 1 mg per mouse on 3 successive days) than among those given the same amount of antibiotic but no cortisone.

Mice infected with Group C streptococcus were treated with aureomycin, streptomycin or terramycin alone and with cortisone acetate. These antibiotics were given subcutaneously twice a day, the first injection being 2-3 hours after infection. Cortisone was injected 1 hour after infection, as in the experiments with penicillin. Results of these experiments are also shown in Table I. It is seen that, in contrast to the results with penicillin, the great majority of streptococcus-infected mice injected with large doses of cortisone and adequate doses of aureomycin, streptomycin or terramycin failed to survive.

Similar experiments were made in which mice infected by i.p. injection of other bacteria were treated with antibiotics known to be effective against them. Dosages of cultures used to infect mice were: *S. pullorum* 1.0 ml, streptococcus (Group A) 0.1 ml; *K. pneumoniae* 0.1 ml of culture diluted 1:30; *P. multicida* 0.1 ml of culture diluted 1:50. Cortisone was first injected 1 hour, and the antibiotics 2-3 hours after infection and treatment was continued for 3 days. In the experiments with *Br. abortus* mice were infected with 0.1 ml of a suspension obtained by washing the growth from a 24 hour culture with 3 ml of saline. The mice in the various groups were treated with cortisone for 5 days and with antibiotics for 7 days starting 24 hours after infection. Survivors were totaled after 2 weeks. None of the untreated controls, or those treated with cortisone, survived this period of observation. Results of treatment of mice infected with the various bacteria and treated with appropriate antibiotics are shown in Table II.

Penicillin given in drinking water protected the majority of mice infected with the Group A streptococcus regardless of whether they received cortisone. However, the therapeutic effect of polymyxin B, streptomycin, neomycin, chloramphenicol or terramycin obtained in mice infected with *K. pneumoniae* was reversed by concomitant treatment with cortisone and *P. multicida, S. pullorum* and *Br. abortus* infections were not controlled when cortisone was given together with the doses of

aureomycin, streptomycin, or terramycin which when used alone insured survival of mice.

Impairment of the effect of antibiotics appears to depend upon the administration of massive doses of cortisone. This is shown by results of treatment of streptococcus infected mice treated with streptomycin, terramycin, and aureomycin (Table I) and by *K. pneumoniae* infected mice treated with terramycin (Table II). If the amount of cortisone given during the 3 day treatment period is decreased below a critical level, reversal of successful treatment by these antibiotics failed to occur.

Discussion. Under the conditions of these experiments, penicillin protects infected mice treated with large doses of cortisone as well as those treated with penicillin alone, and when mice were treated by giving 100 units per ml of drinking water, those which received large doses of cortisone in addition were better protected than those which received no cortisone. Experiments by Mogabgab and Thomas(3) have shown that penicillin in large doses was effective in the treatment of cortisone induced streptococcus infection in rabbits; and Kaplan *et al.*(4) have shown that penicillin prevents deaths due to experimental streptococcal infection in mice exhibiting profound leukopenia as a result of irradiation. The latter authors considered that penicillin, because of its bactericidal action, was effective in the absence of the host's defense mechanisms, and that the lesser effectiveness of aureomycin under these conditions was probably a function of the low dosages employed. In the present experiments, dosages of aureomycin, terramycin, and streptomycin greatly in excess of the amount needed to protect streptococcus-infected mice which received no cortisone failed to protect mice injected with large doses of cortisone. Similarly, these antibiotics and neomycin, polymyxin B and chloramphenicol, when given in doses which protected non-cortisone treated controls failed to protect mice treated with large doses of cortisone against infections caused by various gram negative bacteria. One exception was noted, however, in an experiment in which mice, infected with *K. pneumoniae,* were protected by being treated with 2 mg of streptomycin twice a day although they received cortisone. Most of the cortisone-treated mice which received 1 mg of streptomycin twice a day died, although this amount of streptomycin protected controls. *K. pneumoniae* is notably susceptible to streptomycin and it is probable that at the 2 mg 2 x day level, the infecting organisms are quickly destroyed. In this connection, it should be noted that Miller *et al.*(5) found streptomycin to be the most effective of a number of antibiotics studied in controlling post-irradiation infection.

On the basis of the present experiments, it appears that with the exception of penicillin, the antibiotics studied are unable to complete their chemotherapeutic effect in mice in which normal defense mechanisms have been impaired by large doses of cortisone. Jawetz(6) has briefly described experiments in mice of similar importance in which cortisone interfered more with the protective effect of aureomycin than with that of penicillin.

Summary. The influence of cortisone on the chemotherapeutic effect of antibiotics in experimental bacterial infections in mice has been studied. Cortisone has little or no effect on the protective action of penicillin against streptococcus infections in mice. When administered in doses which regularly protect non-cortisone treated mice, streptomycin, aureomycin, terramycin, chloramphenicol, neomycin and polymyxin B, failed to protect mice treated with cortisone. This reversal of successful antibiotic treatment occurred in mice treated with large doses (0.8 mg) but did not occur in mice treated with small doses (0.2 mg) of cortisone.

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Glycerine as a Stabilizer of Some Complement-Fixing Antigens of Viral and Rickettsial Origin.* (20593)

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In the course of our work we were faced with the problem of preserving the stability of complement-fixing antigens during shipping or long-term storage without refrigeration under unfavorable summer temperatures. Besides, the tendency of the soluble antigens to flocculate after storage in the frozen state and to deteriorate fairly rapidly at plus 4°C presented a serious difficulty. In their paper on the stabilizing action of glycerine on the hemagglutinating capacity of different viruses, Cabasso, Markham, and Cox(1), gave several references on the use of glycerine for the preservation of different biological materials. Hoyle and Fairbrother(2), Enders and coworkers(3), Shepard(4), and other authors have reported on the flocculation of influenza, mumps, and typhus antigens of the soluble types at different temperatures, and have discussed the influence of pH, agitation, dilution and other factors both on the formation of turbidity and on the serologic activity of the antigens.

We tested the stabilizing action of glycerine on the complement-fixing capacity of the following antigens: Q Fever (Henzerling), Q Fever (Nine Mile), Typhus Fever, epidemic (soluble type), Typhus Fever, murine (soluble type), all 4 made by Lederle Laboratories, U.S.A.; influenza A-prime (soluble type), Influenza A (soluble type), Influenza B (soluble type) antigens, all made from chorioallantoic membranes in our laboratory; and Mumps and LCM antigens. The influence of glycerine in preventing the visible flocculation of heated antigens was also investigated.

Materials and methods. Glycerine and saline antigens. The glycerine solutions were made up volumetrically, usually in 2-ml amounts as follows: to one ml of the antigen, either undiluted glycerine or a 40% glycerine solution in saline (Glycerin, Konc., rein D 1, 25-26 (Bayer)) was added up to the 2-ml mark of calibrated narrow test tubes. In that way 1:2 dilutions of the antigens were obtained which contained 50 or 20% glycerine. The control antigens were made in a similar fashion, substituting saline for glycerine. *Titration method.* If not stated otherwise, the antigens were titrated in serial 2-fold dilutions against about 8 units of the homologous human convalescent serum. The complement-fixation technic followed that used at the Virus Reference Laboratory, Colindale (Director, Dr. F. O. MacCallum)(5). The volume unit was 0.1 ml. The complement is titrated for each lot of sensitized cells (2% cells with 2 units of hemolysin) and the 2 units of complement to be used in the test are strictly controlled (control-tubes are set up to contain 2, 1, ½, and ¼ units of complement, respectively). All antigens (except LCM) were tested by 90-minutes fixation at 37°C, addition of sensitized red cells and a second incubation of 30 minutes, with one shaking after the first 5 minutes. For the LCM antigen, overnight fixation at 4°C was used before the addition of the sensitized cells. Controls of serum, antigen, sensitized cells and the exact amount of complement used in the test were included in each experiment. Dilutions of serum samples used in comparative studies were made in large quantities after the usual inactivation for 30 minutes at 56°C, and before distribution in the respective sets of test tubes. *Heating of antigens.* The antigen solutions were heated in sealed ampoules of thin, neutral glass, containing usually one-ml amounts. During heating, the ampoules were held under water level in either a water-bath with well controlled temperature, in boiling distilled water or in the autoclave. The heated ampoules were shaken thoroughly before opening. *Recording of results.* Results of the complement-fixation tests were recorded as 3, 2, 1, ± or negative. These were used to designate absence of

* The technical assistance of Mrs. Marthe Milivojevich is gratefully acknowledged.

TABLE I. Heat Resistance of Typhus Antigens (Epidemic, Soluble Type).

Antigens	Min.	Reciprocals of the titers of Saline-antigens	Glycerine-antigens
Unheated		32	32
Heated at 56°C	30	16	32
	120	8 (16++)	32
" 100°C	10	4	8
	30	2	8
	80	1–	8
	120	1–	4

hemolysis, or 25, 50, 75, and 100% hemolysis, respectively. End points of titrations are expressed by the reciprocal of the highest antigen-dilution showing 3 or 2+ fixation (25% hemolysis or less). Results recorded as 16, 16++, 16+, 16±, or 16— are to be respectively interpreted as 3, 2, or 1+, ± or negative at a 1:16 antigen dilution. In some instances, the formation of turbidity and the degree of flocculation of both heated saline and glycerine preparations were recorded.

Q Fever antigen (Henzerling). Upon testing the heat resistance of both the saline and glycerine preparations at different temperatures, surprising stability of Q Fever antigen was observed. After heating at 56°C for 270 minutes, at 100°C for 160 minutes, or even after autoclaving at 110°C for 30 minutes, no change in titers of the heated antigens could be detected when compared with unheated preparations. The only difference was that after heating both antigens at 120°C for 30 minutes, a flocculation developed in the saline antigen while the glycerine antigen remained unchanged.

Typhus fever antigen (epidemic, soluble type). Table I illustrates the sensitivity of the saline antigen which was rapidly lost during heating in boiling water: an 8-fold drop in the complement-fixing titer took place in 10 minutes at 100°C, whereas boiling for 80 minutes completely destroyed its complement-fixing ability. In contrast, glycerinated antigen maintained a fair titer after being exposed to the same temperature for the same length of time. Table II illustrates the results of 4 checkerboard titrations of both the saline and glycerine antigens before and after heating for 30 minutes at 100°C. Both preparations were titrated against the different dilutions of the same serum. Following exposure to 120°C for 30 minutes, the saline antigen showed a marked flocculation, whereas the glycerine antigen remained quite clear.

Influenza A-prime (strain FM 1 antigen). Table III illustrates well the stability of influenza antigen after exposure to 56°C for different lengths of time. Complete destruction of activity of the saline antigen was observed after 30 minutes, whereas the glycerine antigen remained unchanged even after 2 hours of heating. In addition, Influenza A-prime antigens containing 0, 20, and 50% glycerine, respectively, were prepared in 4-ml amounts. Each of the 3 antigens was divided into 2 equal portions. The first was kept in well-stoppered tubes in the incubator at 37°C, and the second was stored in icebox at +4°C. After different time intervals aliquots from

TABLE II. Checkerboard-Titrations of Typhus Antigens.

Dilutions of serum	Dilutions of unheated antigens: Saline-antigen 1/2	1/4	1/8	1/16	1/32	Glycerine-antigen 1/2	1/4	1/8	1/16	1/32
1/100	3	3	3	3	1	3	3	3	3	2
1/200	1	3	3	3	±	2	3	3	3	±
1/400	—	—	1	2	±	—	—	2	2	±
1/800	—	—	—	—	—	—	—	—	—	—
	Dilutions of antigens heated for 30 min. at 100°C: Saline-antigen 1/2	1/4	1/8	1/16	1/32	Glycerine-antigen 1/2	1/4	1/8	1/16	1/32
1/25	1	±	—	—	—	3	3	3	1	—
1/50	±	—	—	—	—	3	3	3	—	—
1/100	—	—	—	—	—	2	1	—	—	—
1/200	—	—	—	—	—	—	—	—	—	—

TABLE III. Heat Resistance of Influenza A-prime Antigens.

Antigens	Min.	Reciprocals of the titers of Saline-antigens	Glycerine-antigens
Untreated		32 (64+)	64
Heated at 56°C	10	16 (32+)	64
	30	2–	64
	60	2–	64
	120	1–	64++

the 6 samples of antigen were titrated against portions of the same serum, which was stored in the frozen state in small amounts at all times. The serum was always inactivated just before use.

Table IV shows the results obtained during a 115-day observation period. All 3 samples of antigens show very similar behavior when kept at +4°C, with a slightly slower rate of deterioration of the glycerine preparation when compared with the saline antigen. The saline antigen, when kept at 37°C for periods longer than one month, completely loses its activity, whereas with 50% glycerine it retains its titer fairly well under the same conditions. A greater rate of deterioration took place in the sample containing 20% glycerine, which was somewhat more stable, however, than the saline control.

Table V presents the data on the flocculation observed in both saline and glycerine antigens after exposure to different temperatures for different time intervals. The activity of the saline antigen exposed to a temperature of 80°C for one minute was completely destroyed, while the glycerine antigen showed just about a 2-fold decrease in titer following the same treatment.

Other antigens. Experiments similar to those described above were also performed with other antigens. A pronounced protective effect of glycerine on the heat stability and keeping quality of the following antigens was observed: Typhus-fever antigen (murine, soluble type), made by Lederle Labs., U.S.A.; influenza A antigen (strain PR8, soluble type), influenza B antigen (strain Yugoslavia B 1, soluble type), and LCM antigen (soluble type, prepared from infected guinea pig spleens), all 3 prepared in our laboratory. A very marked effect of glycerine in preventing flocculation after exposure to raised temperatures was also observed with these 4 antigens. The commercial Q Fever antigen (Nine Mile strain, made by Lederle Labs.) was tested in the same way as the Henzerling antigen, and was found to possess the same remarkable heat stability. However, glycerine was of value in preventing flocculation of the Nine Mile Q Fever antigen after autoclaving for 30 minutes at 110°C or 120°C. The stability of mumps antigen of the viral type was also investigated, using either a commercially available antigen (Lederle Labs.) or crude virus infected allantoic fluid prepared in our laboratory. No reproducible results were obtained in a few experiments with either of the 2 mumps antigens tested.

It might be of interest to report that 50% glycerine was also found capable of preventing both flocculation and deterioration in the tested samples of soluble antigens (*e.g.*, LCM), which tended to flocculate after having been stored at temperatures of about —30°C.

Discussion. In the experiments described above, no pH determinations were made on

TABLE V. Appearance of Influenza A-prime Antigens after Heating.

Heating Time, min.	Heating °C	Saline-antigen	Glycerine-antigen
20	56	turbidity	unchanged
120	56	flocculation	"
1	80	coagulation	slight turbidity
1	100	"	coagulation

TABLE IV. Resistance of Influenza A-prime Antigens.

% of glycerine in the antigens	Kept at, °C	Time in days 0	1	3	6	12	19	35	69	115
0	4	32	16	16++	8	8	8	8	8	8
0	37	32	8	8	8	4	4	2++	0	0
20	37	32	16+	16++	8	8	8	8++	8	4
50	37	32	16++	16	16++	16	16+	16++	16+	8

the antigens prior to heating. The amount and dilution of the antigens exposed to heating, as well as titer and protein content of the different samples of the same antigen, were not kept strictly constant when repeating the same experiments.

The results reported refer to single experiments, but very similar, if not identical, results were obtained repeatedly with the same kinds of antigen, under the same conditions. The only exception was experienced when testing mumps antigens, with which no reproducible results were obtained under the conditions used.

Although glycerine was found to have a protective effect against flocculation of Q Fever antigen at higher temperatures, glycerinated preparations do not seem to present any advantage because of the remarkable heat stability of this antigen even in the absence of glycerine.

Glycerine alone or in combination with any of the antigens tested, as well as glycerine mixed with positive or negative sera, consistently failed to give either pro- or anti-complementary effects.

After autoclaving for 30 minutes at 110°C or 120°C, both saline and glycerinated preparations of some antigens (*e.g.*, Nine Mile Q Fever antigen) became somewhat anti-complementary. However, the anti-complementary level never reached that of the specific antigen titer and since it appeared only after exposure to temperatures higher than 100°C, it seems evident that it does not add to the problem at hand.

The results obtained seem to justify therefore the addition of 50% glycerine as preservative to the complement-fixing antigens tested so far in our laboratory.

Summary. The stabilizing action of glycerine on some complement-fixing antigens of viral and rickettsial origin was investigated. Its ability to prevent rapid deterioration of antigen-titers and visible flocculation following long storage or exposure to unduly high temperatures has been reported.

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Binding of Histamine by Animal Proteins. (20594)

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Recent reports(1,2) have given evidence for binding of histamine by components of normal human blood serum, especially γ-globulin. The availability of C^{14}-histamine* made it possible to check these results by the dialysis-equilibrium technic(3).

Guinea pig lung has been reported(4) to contain relatively high concentrations of histamine. However, it was demonstrated(5) in experiments with isotopically labeled histamine and *L*-histidine that injected histamine is rapidly metabolized and excreted in the urine by guinea pigs and does not appear to any appreciable extent in the internal organs, whereas injected *L*-histidine is converted to histamine which is present in the organs for long periods. It was therefore of interest to determine whether added C^{14}-histamine would be bound by guinea pig lung homogenates or equilibrate with histamine already present.

Experimental. Human plasma proteins†

* We are grateful to Dr. R. W. Schayer of this Institute for supplying the labeled histamine.

† These human plasma fractions were generously furnished by Dr. E. J. Cohn of Harvard University Medical School.

were dissolved in Tyrode solution, dialyzed against Tyrode overnight in a cold room, and finally diluted to a concentration of 0.5% of the protein as weighed. Normal human blood serum was dialyzed against Tyrode solution and diluted with Tyrode to 3.3 times the original volume of serum. Guinea pig lung was homogenized with Tyrode solution in a Potter-Elvehjem homogenizer, dialyzed, and diluted to 1% by weight of wet tissue. Aliquots (10 ml) of each solution or suspension were placed in cellulose bags in test tubes containing solutions of 1 mg, 10.4 γ, and 0.4 γ of C^{14}-histamine in 11 ml of Tyrode solution, each having the same total radioactivity. Identical tubes were set up simultaneously containing only Tyrode solution inside the cellulose bags. Each protein solution or suspension was equilibrated against each concentration of histamine by shaking the stoppered tubes for 18-20 hours in a cold room (2-5°C), all experiments being run in duplicate. Portions (10 ml) of the solutions outside the bags were then added to 66.4 mg of histamine dihydrochloride carrier, acidified with HCl, evaporated to dryness, and converted to picrates. The picrates were dissolved in aqueous alcohol, treated with norit, recrystallized, plated in stainless steel cups, and counted to 5% accuracy at infinite thickness in a flow counter. Under these conditions the C^{14}-histamine in each tube had a total activity of 200 c.p.m. above background. If the histamine became distributed uniformly, each 10 ml portion of solution should have had an activity of 95 c.p.m. above background under the conditions used. Within experimental error this was the radioactivity found not only in the tubes containing only Tyrode solution inside the bags, but also in the tubes containing 0.5% solutions of human γ-globulin, β_1-globulin, and plasma albumin as well as normal human serum and the 1% guinea pig lung homogenate. No evidence was obtained for binding of histamine at equilibrium levels of 47.6, 0.5, and 0.02 γ per ml by these proteins.

The above experiments were performed under different conditions than those used by the previous workers(1,2). For γ-globulin more comparable experiments were carried out. The bags contained 4 γ of histamine (2000 c.p.m. above background when assayed for radioactivity as above) and 5 mg of γ-globulin in 10.2 ml of Tyrode solution and were placed in 5.1 ml of Tyrode containing 2 γ of histamine (1000 c.p.m.). As above, controls were run in which γ-globulin was absent. The tubes were shaken 3 hours at room temperature (26-8°C), and 1.0 ml aliquots of the solutions outside the cellulose bags were assayed for radioactivity. The histamine was uniformly distributed in the tubes equilibrated with γ-globulin, as well as the controls, all samples assaying 195 c.p.m. above background per ml (0.39 γ/ml) within the counting error of 5%. There was therefore no evidence for binding of histamine by γ-globulin under these conditions. The solutions both inside and outside the cellulose bags were checked for their histamine content by pharmacological assay on guinea pig intestine.‡ Within experimental error the solutions outside the bags contained the equilibrium concentration of histamine (0.45 γ/ml), but the solutions within the bags containing γ-globulin gave low assays (0.25 γ/ml). The latter levels were increased by acid hydrolysis or trichloroacetic acid precipitation of the protein, but the experimental uncertainty was greater than any uptake of histamine which might have occurred.

An attempt was also made to demonstrate binding of histamine at room temperature by a much higher level of γ-globulin. The solutions within the cellulose bags contained a 5% solution of the protein and the concentration of labeled histamine was 0.33 γ/ml (163 c.p.m. per ml by the usual assay procedure). Both the solution equilibrated with protein and that equilibrated with Tyrode assayed as expected for uniform distribution of histamine on both sides of the cellulose membrane.

Discussion and summary. The results with labeled histamine show that it is not bound reversibly by blood proteins. As found by Parrot *et al.*(1,2), γ-globulin causes a diminution of the pharmacological activity of hista-

‡ Dr. G. Ungar of this Institute kindly carried out these assays.

mine solutions, but on the basis of the dialysis-equilibrium experiments this effect cannot be attributed to binding of histamine by the protein. Schayer(5) has pointed out the non-equilibration of guinea pig organs with exogenous histamine *in vivo*. The present results show that *in vitro* guinea pig lung is incapable of binding added histamine or of equilibrating added with bound histamine. Apparently, binding of histamine requires formation at the binding site.

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Anti-mycobacterial Properties of a New Derivative of Isoniazid. (20595)

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Since the first description of the marked anti-tuberculous properties of isoniazid(1,2,3, 4) and iproniazid, it has been shown that derivatives of isoniazid in which one or both of the terminal hydrogens have been replaced by substitutions might possess similar high activity both *in vitro* and *in vivo*(5,6,7,8). Of the numerous compounds of this type synthesized by Dr. H. H. Fox in the Roche Chemical Research Laboratories, which were studied for their activity against *Mycobacterium tuberculosis* and were found to exert the expected effect, one substance appeared to have favorable properties of both toxicity and chemotherapeutic activity. This substance, 1,1′-methylenebis(2-isonicotinylhydrazine) from now on designated as Ro 2-4969, which contains 2 isoniazid molecules linked by the methylene group is characterized by the lack of solubility in water, alcohol and most other organic solvents. Despite this physical property which distinguishes it sharply from other active members of the hydrazine series, it exhibited an effect comparable to the parent substance. The results of the experimental studies carried out with Ro 2-4969 are reported in the present note.

Materials and methods. The H37Rv strain of *M. tuberculosis* obtained through the courtesy of Mr. W. Steenken, Jr., of Trudeau Sanatorium on December 20, 1947 was used in all experiments. Adult albino mice of 18-20 g from our own colony were used throughout the investigation. The technics of the experiments in mice used in the evaluation of Ro 2-4969 are identical with those described in earlier publications(2,9) and are based on the effect of a 21 day treatment with medicated diet and examination of the lungs both immediately after discontinuation of treatment as well as 21 days after termination of therapy. The lack of solubility of Ro 2-4969 prompted also experiments in which small pellets were inserted in the subcutaneous tissue. This was followed by intravenous infection with *M. tuberculosis* H37Rv at different intervals after the implantation. Further details of the procedure are given in the text.

Toxicity for mice. Acute toxicity tests carried out by administering a single dose of the compound by gavage gave an LD_{50} of 3900.0 mg/kg.*

In vivo experiments with M. tuberculosis. 1. *Effect of oral drug administration.* If medicated diet with Ro 2-4969 was started immediately after the intravenous infection with strain H37Rv and continued for 21 days at which time the mice were sacrificed and examined, the majority of animals were found to be protected at a dose of 25.0 mg/kg/day

* Experiments in different species of animals including chronic toxicity tests have been carried out by Dr. Benson and associates in the Roche Pharmacology Laboratory and will be reported elsewhere.

TABLE I. Antitubercular Activity of Ro 2-4969 in Intravenous Infection of Mice with Strain H37Rv. Infection: 0.5 ml of a 10^{-1} dilution of a 7- to 10-day-old culture in Dubos' medium. Treatment: Medicated diet for a 21-day period.

Daily dose, mg/kg	No. of mice	Mice protected No.	Mice protected %	No. neg. cultures / No. cultures taken
25.0	40	39	97.5	21/27
12.5	19	6	31.6	1/19
6.25	20	0	0.0	4/20
Controls	39	0	0.0	3/39

TABLE II. Antitubercular Activity of Ro 2-4969 in Intravenous Infection of Mice with Strain H37Rv. Infection: 0.5 ml of a 10^{-1} dilution of a 7- to 10-day-old culture in Dubos' medium. Treatment: Medicated diet for 21 days. Normal diet for 21 days.

Daily dose, mg/kg	No. of mice	Mice protected No.	Mice protected %	No. neg. cultures / No. cultures taken
500	17	16	94.1	10/14
250	20	8	40.0	11/20
125	17	10	58.8	5/14
50	10	3	30.0	3/10
Controls	17	0	0.0	1/9

(Table I). These mice also showed the phenomenon of lack of growth of cultures taken directly from lung tissue. This observation which seems to be characteristic for isoniazid and related compounds(1) has meanwhile been observed by other investigators (10,11,12) with isoniazid. The 50% protective dose (PD_{50}) as calculated according to Reed and Muench was found to be 15.3 mg/kg/day for Ro2-4969. If the observation period was prolonged for an additional 3 weeks without therapy, the low doses used in the first group of experiments could be expected to be inactive since isoniazid required doses of more than 50.0 mg/kg/day for lasting protection(2). This indeed was the case and a dose of 125.0 mg/kg/day or more of Ro 2-4969 was necessary to produce lasting protection (Table II). The PD_{50} for this type of experiment was calculated to be 171 mg/kg/day. The higher dose of 250.0 and 500.0 mg/kg/day also showed absence of viable bacilli in at least 70% of the animals cultured. That this observation does not constitute a claim of "sterilization" of the lung tissue has been mentioned in earlier papers(1,2).

2. *Protective effect of implanted pellets.* Small pellets containing 25.0 mg of Ro 2-4969 which were prepared for us by Dr. L. Magid of the Roche Pharmaceutical Laboratories, were implanted in the dorsal subcutaneous tissue of mice. While in the majority of animals the pellet caused no untoward effects, some animals showed signs of necrotization or at least loss of hair at the site of implantation. The pellets were, however, palpable for periods of 2-3 weeks. At intervals of 0, 3, 7, 14, 21 and 42 days following implantation of the pellet, the animals were infected with *M. tuberculosis* H37Rv intravenously. Each group of animals originally consisting of 10 was sacrificed on the 21-25th day after infection and the lungs examined for the presence or absence of gross lesions.

The results of these experiments (Table III) indicate that marked protection was conferred on the majority of animals infected within the first 3 days after the implantation of the pellet. Even after 7 days 50% of the animals were free of lesions. If 2 weeks or longer had elapsed, no appreciable protective effect was observed. The slow release of Ro 2-4969 from the site of implantation was seemingly sufficient to produce an effect similar to the continuous drug absorption from the intestinal tract even to the extent that also about 60% of the animals which were infected within 3 days after pellet implantation showed negative cultures from their lungs. One can, therefore, characterize the effect of the implantation of the pellet by stating that a sufficient amount of the compound seems to be released for a period of about 25 days so as

TABLE III. Protective Effect of Pellets of Ro 2-4969 in Intravenous Infection of Mice with Strain H37Rv. Infection: 0.5 ml of a 10^{-1} dilution of a 7- to 10-day-old culture in Dubos' medium. Treatment: 25 mg pellet implanted subcutaneously.

Insertion of pellets .... days before infection	Total observation (days)	Protection rate*	No. neg. cultures / No. cultures taken
0	25	9/9	5/9
3	24	8/9	5/8
7	28	5/10	1/10
14	35	3/10	1/10
21	42	0/10	1/9
42	63	0/9	0/8
Controls	24–25	0/20	1/20

* No. of mice free of lesions/No. of treated mice.

to inhibit the lesions in a majority of animals and even to interfere with the recovery of the causative organism.

Discussion. The experiments presented seem to indicate that the insoluble isoniazid derivative, 1,1′-methylenebis(2-isonicotinyl hydrazine), is apparently absorbed both from the gastro-intestinal tract and the subcutaneous tissue in sufficient amounts to exert a marked protective effect in the intravenous *M. tuberculosis* infection of mice. Since a method of isoniazid determination can be used successfully for the determination of blood concentrations of this compound(13), one might assume that a breakdown to isoniazid takes place in the body.

It is, therefore, also not surprising that the activity of this substance is fundamentally the same as that of isoniazid. Due probably to its slower and perhaps less complete distribution, the activity appears to be about 3-4 times less than that of isoniazid. The low toxicity results in a more favorable chemotherapeutic ratio of an LD_{50}/PD_{50} of 255 as compared to 44.1 for isoniazid(1). The assumption that a compound like Ro 2-4969 with its still quite appreciable anti-tuberculous activity might be suitable for producing a therapeutically favorable slow absorption appropriate to build up a consistently high blood level seems to be born out by the investigations of Larson and Dickie(13).

It might also be mentioned at this point that preliminary experiments indicate that rats infected with *M. lepraemurium* and kept on a diet of 0.4% Ro 2-4969 for a period of 4 months failed to develop any lesions even 5 months after discontinuance of the medicated diet.

Summary. (1) Chemotherapeutic experiments with a new insoluble derivative of isoniazid, 1,1′-methylenebis(2-isonicotinyl hydrazine), are described which show that this compound which was of very low toxicity possessed marked anti-tuberculous properties in mice infected with *M. tuberculosis* H37Rv. (2) This substance was evidently absorbed from the intestinal tract as well as from the subcutaneous tissue where it was implanted in the form of a pellet. (3) Although the new compound was found to be 3-4 times less active than isoniazid on the basis of the absolute value obtained for the 50% protective dose, the ratio LD_{50}/PD_{50} was very favorable (255) due to the low toxicity.

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Study of Relationship between pH of Urine and Sodium and Potassium Excretion.* (20596)

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A normal, characteristic pattern of diurnal pH variation has been recognized since the middle of the last century(1), but to date its occurrence has not been satisfactorily explained. Hubbard and Steele(2) found that awakening in the morning caused a rise in urinary pH. This "alkaline tide" is independent of meals. Ryberg(3) observed a drop in pH immediately following a meal. Two to 4 hours after a meal, urinary pH rises(4,5). The secretion of gastric juice, changes in respiration, as well as secretion of pancreatic juice have all been factors considered important in this phenomenon. Stanbury and Thomson(6) have shown a clear parallelism between urinary pH, sodium, potassium, chloride and bicarbonate. They also found that diurnal excretory rhythm persisted in the face of starvation, water deprivation, salt deprivation, sustained action of desoxycorticosterone, pituitary antidiuretic hormone, and temporary disturbance of the sleep rhythm.

In the course of a study on the effect of adrenalin on the kidneys(7,8), it was found that a patient with a low sodium output showed a lack of diurnal variation in the pH of urine. The addition of salt to this patient's diet resulted in increased sodium output and restored a completely normal pattern of alkaline tides. This observation prompted a series of experiments concerned with urinary sodium and potassium excretion and their relation to the variation in urinary pH.

Methods. Twenty-three male medical students and 23 postoperative patients were used in this study. Serum sodium was determined in nearly all subjects. The students voided once every hour for at least 8 hours (8 A.M. to 4 P.M.) and the urine was analyzed for sodium, potassium, ammonia, chlorides and titratable acidity. The pH was determined immediately on the freshly voided samples but no precautions were taken to avoid CO_2 loss. In all the patients, an indwelling catheter was placed in the bladder and continuous recording of the pH of the urine was carried out for a minimum period of 8 hours (8 A.M. to 4 P.M.). A recording pH meter which has been described previously(7,8), or a smaller portable unit,† was used for the continuous determination of pH. The urine samples from the patients were collected every hour and the pH was determined on these pooled samples without attempting to prevent the loss of CO_2. Since the continuous determination of pH took place in a closed system, pH differences between the urine in the closed and open system would have indicated the CO_2 loss which had taken place. One would expect a difference in the magnitude of the pH variations using the 2 different methods of collection. If an increase and a decrease in pH occurred within an hour, a pH determination on samples collected hourly would tend to nullify the variations. Nevertheless, a comparison of the 2 methods over a minimum period of 8 hours, showed that a lack of variation seen with one method, would also be revealed by the other. Furthermore, the degree of variation in pH using the continuous recording system, compared fairly well with that of the determination on the samples collected every hour. Since this study deals with the degree of pH variation over a minimum period of 8 hours, this variation will, for convenience, be expressed as the standard deviation of the pH's of the samples collected hourly. Thus, a standard deviation of 0 to ± 0.2, indicated a lack of normal variation in the pH of the urine. Normal diurnal variations result in a higher standard deviation. Sodium and potassium in

* This investigation was supported with funds from the Dr. Henry C. Buswell Memorial and the Office of Naval Research.

† The Macbeth Corp. Newburgh, N. Y., has generously cooperated with us in making a small unit designed for the purpose of continuously reading the pH of urine.

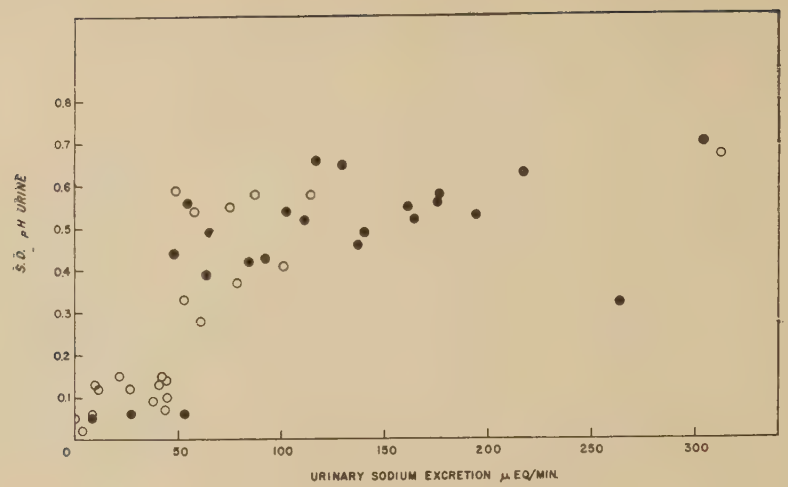

FIG. 1. Correlation of urinary pH variations with mean sodium excretion per min. for a minimum period of 8 hr. Variations in urinary pH are expressed as stand. dev. and it can be seen that 16 patients had very little, or no variation in pH of urine. Circles indicate patients, black dots represent normal subjects.

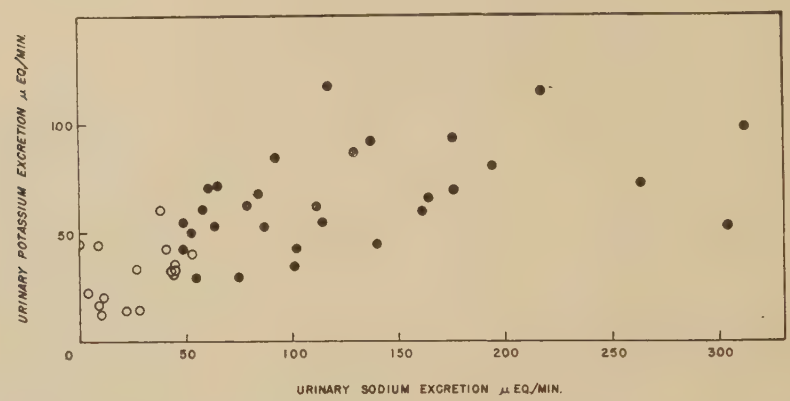

FIG. 2. Correlation between mean excretion of potassium per min. and mean excretion of sodium per min. during a minimum period of 8 hr. Circles represent all cases in which minimal or lack of urinary pH variation was seen, black dots represent the cases exhibiting normal variations.

the hourly urine samples as well as in serum were determined using a Beckman Spectrophotometer model DU with flame photometer attachment. The urine samples were also analyzed for chloride(9), titratable acid(10) and ammonia(11).

Results. In Fig. 1, variation in the pH of the urine expressed as standard deviation of pH is plotted against urinary sodium excretion per minute. It can be seen that when sodium excretion drops below approximately 45 microequivalents per minute, normal pH variation is not observed. Fig. 2 shows the relationship between the mean excretion of sodium and the mean excretion of potassium per minute. In most instances when the standard deviation of the pH of the urine was low, the mean excretion per minute of potassium was also below 45 microequivalents. This figure also shows that only in one of 16 cases with low pH fluctuation was the mean sodium excretion marginally above 45 microequivalents per minute. Of the cases with a potassium excretion below 45 microequivalents per minute, 5 had a normal urinary pH variation.

An attempt to correlate the pH variations with the mean excretion per minute of ammonia and titratable acidity yielded no definite

relationship between either of the two. However, all the subjects exhibited an excretion of titratable acid within normal range.

All of the 16 subjects which exhibited a relatively fixed urinary pH with no or only minimal variation, had an acid urine. However, the actual pH varied considerably from individual to individual (4.8-6.0). Therefore, no correlation was found between the sodium excretion and the actual pH of the urine.

Discussion. The mechanism accomplishing acidification of the urine, according to Pitts (12,13), involves the carbonic anhydrase catalyzed hydration of carbonic acid and a process of ion exchange. Since the administration of the carbonic anhydrase inhibitor 6063 (Diamox) can result in a sodium diuresis which is greatly in excess of that which could be accounted for in terms of hydrogen ion exchange, it must be assumed that carbonic anhydrase is responsible in some additional way for a much greater reabsorption of sodium than has hitherto been assumed. This has recently been suggested by Pitts(14).

If this is true, it would follow that maximum activity in the systems involving carbonic anhydrase should result in maximum reabsorption of sodium and maximum production of hydrogen ion, the latter probably being responsible for the fixation of urinary pH on the acid side.

The results obtained in this study indicate that a fixed acid urine is concomitant with a high reabsorption of base. This would be expected if the above mentioned theory is correct.

The data in this paper have been utilized clinically to evaluate excretion of base. It has been a constant observation that patients exhibit water and salt retention as long as they maintain a fixed urinary pH provided that titratable acidity is not exceedingly high (above 25 microequivalents per minute). This has repeatedly been observed in cases of traumatic shock. In cases where it has been possible to restore normal pH fluctuations, this has been concomitant with sodium diuresis leading to water and salt balance. Much work remains to be done along these lines but so far it has been possible to utilize this technic with great advantage in some severely burned patients(15). It is worthwhile to emphasize that only patients with supposedly normal kidneys have been considered and it is doubtful whether the correlation described in this study will have bearing in patients with renal damage.

Summary. Urinary pH was determined hourly for a minimum period of 8 hours in 46 cases. The urine samples which were collected every hour were analyzed for sodium, potassium, ammonia, chloride and titratable acidity. A definite correlation between sodium-potassium excretion and variation in pH of the urine was established. All 16 exhibiting a sodium excretion below 45 microequivalents per minute had a fixed urinary pH at a level which differed considerably from individual to individual. Only one person with a slightly higher sodium excretion (53 microequivalents per minute) had a fixed urinary pH. Potassium showed a similar pattern although less consistent, since 5 subjects with normal pH variations had a low potassium excretion.

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Action of Fluoroacetate on Uptake of PAH by Renal Slices of the Dog.* (20597)

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In a previous publication(1) we have shown that treatment of the anesthetized dog with sodium fluoroacetate inhibited renal p-aminohippurate (PAH) secretion as well as glomerular filtration rate and blood flow through the kidney(1). These hemodynamic changes made interpretation rather difficult and it was deemed desirable to determine the effects of fluoroacetate on PAH secretion by the method of Cross and Taggert(2). Cross and Taggert(2) have shown that the slice:medium ratio of PAH is an index of the secretory activity of the renal slices. In most of our experiments both the slice:medium ratio (S:M ratio) for PAH as well as the oxygen consumption have been determined in the presence and absence of acetate as substrate.

Methods. Dogs weighing 6 to 14 kg were anesthetized with 30 to 35 mg per kg of pentobarbital given intravenously. The left kidney was removed first and the S:M ratio and oxygen consumption were determined. The animal then received an infusion of isotonic saline, 3.4% sodium bicarbonate or 2 or 6% sodium acetate containing pentobarbital (50 mg/l.). The rate of infusion was about 10 cc /minute/m^2 body surface. About 30 minutes after the infusion was started either saline or fluoroacetate was injected intravenously. About 3 hours after this injection the right kidney was removed and the oxygen consumption and PAH slice:medium ratio were determined. The same technic as described by Cross and Taggert(2) was used except that the PAH concentration was 0.0013M. In some of the experiments the slices were incubated in a Dubnoff metabolic shaking machine and the gas phase used was 100% oxygen.

Results. Control experiments have shown that the oxygen consumption and the slice:medium ratio of PAH of the left and right kidney were about the same. The procedure used was the same as for the fluoroacetate experiments except that saline was given instead of fluoroacetate (Table I). From Table II it can be seen that the higher dosages of fluoroacetate depressed both oxygen consumption and PAH uptake in the absence of substrate and completely eliminated the increment in PAH uptake and oxygen consumption produced by acetate. With doses of 0.5 and 0.25 mg of NaFAc per kg the influence on oxygen consumption and PAH uptake in the absence of substrate was minimal but the increment produced by acetate as substrate, was practically completely eliminated. Table I shows the results when acetate was infused. Acetate infusions alone increased both oxygen consumption and the S:M ratio. The infusion of acetate protected the kidney against fluoroacetate and this protection was roughly proportional to the rate of infusion of sodium acetate (Table III). Control experiments with bicarbonate infusions did not show any protection against NaFAc and are included in Table III.

Discussion. It is apparent from the above findings that the metabolic reactions involved in the increment in oxygen consumption and S:M ratio produced by acetate are far more sensitive to fluoroacetate poisoning than the metabolic reactions occurring in the absence of acetate. It must be concluded that fluoroacetate primarily blocks those metabolic pathways related to acetate oxidation but is less effective in blocking the endogenous metabolism. Fluoroacetate resistant energy producing mechanisms have been demonstrated in intestinal(3-5) and in uterine(6) smooth muscle. It is possible that the kidney may have such a fluoroacetate resistant pathway thus explaining the relative resistance of the endogenous oxygen consumption and PAH uptake to NaFAc in the absence of added ace-

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TABLE I. Influence of Saline and Acetate Infusions on Oxygen Consumption and PAH Uptake of Dog Kidney Slices. Saline and acetate infusions about 10 cc/min./m² body surface. Left kidney (control) removed first. Three hours after start of infusion right kidney removed.

	Left kidney (control)				Right kidney (experimental)			
	Oxygen consumption		PAH S:M ratio		Oxygen consumption		PAH S:M ratio	
Infusion, %	No substrate added	NaAc, .01 M	No substrate added	NaAc, .01 M	No substrate added	NaAc, .01 M	No substrate added	NaAc, .01 M
.9 NaCl	.984	1.531	4.4	12.7	.960	1.576	4.2	11.8
" "	.781	1.370	3.6	9.5	.792	1.394	3.7	9.0
2 NaAc	—	—	3.59	8.76	—	—	7.2	8.0
" "	.683	1.130	3.3	7.4	.931	1.24	6.4	8.3
6 NaAc	.912	1.473	3.2	9.6	1.420	1.400	9.4	9.2
" "	1.02	1.625	4.0	11.5	1.715	1.532	10.8	12.0
" "	—	—	4.3	10.5	—	—	12.3	11.5
" "	—	—	4.2	10.4	—	—	9.11	9.4

TABLE II. Influence of Sodium Fluoroacetate on Oxygen Consumption and PAH Uptake of Dog Kidney Slices. Saline infusion about 10 cc/min./m² body surface. Left kidney removed first as control. Three hours after injection of saline or fluoroacetate right kidney removed.

	Left kidney (control)				Right kidney (experimental)			
	Oxygen consumption		PAH S:M ratio		Oxygen consumption		PAH S:M ratio	
Dosage of NaFAc, mg/kg	No substrate added	NaAc, .01 M	No substrate added	NaAc, .01 M	No substrate added	NaAc, .01 M	No substrate added	NaAc, .01 M
0	.984	1.531	4.4	12.7	.960	1.576	4.2	11.8
0	.781	1.370	3.6	9.5	.792	1.394	3.7	9.0
.25	1.04	1.632	6.8	17.2	1.00	1.18	6.45	6.8
.25	.845	1.276	4.0	9.7	.800	.901	4.2	6.05
.25	.712	1.140	3.7	8.6	.760	.73	3.5	4.0
.5	1.19	1.570	7.0	16.07	1.00	.925	3.10	2.45
.5	.69	1.21	4.0	11.70	.71	.820	3.14	4.54
.75	.915	1.31	2.6	5.43	.785	.790	1.4	1.59
1	.884	1.28	3.5	9.86	.715	.731	2.3	2.01
1	.904	1.465	3.9	10.70	.76	.742	1.85	2.05
2	.773	1.211	4.4	13.5	.525	.510	1.93	1.6

TABLE III. Influence of Sodium Acetate Infusions on Inhibition of Oxygen Consumption and PAH Uptake Produced by Sodium Fluoroacetate. Left kidney was removed first and used as control. Infusion of sodium acetate or bicarbonate was started (about 10 cc/min./m²) and 30 min. later 1 mg sodium fluoroacetate/kg given. Three hours after fluoroacetate administration right kidney removed and studied.

	Left kidney (control)				Right kidney (experimental)			
	Oxygen consumption		S:M ratio		Oxygen consumption		S:M ratio	
Infusion, %	No substrate needed	NaAc, .01 M	No substrate needed	NaAc, .01 M	No substrate needed	NaAc, .01 M	No substrate needed	NaAc, .01 M
NaAc 2	.795	1.331	4.1	11.5	1.390	1.415	6.4	8.2
2	.937	1.674	3.5	8.2	1.610	1.572	4.8	6.5
6	.882	1.248	3.2	9.6	1.331	1.295	10.5	9.3
6	.768	1.310	2.9	9.0	1.285	1.335	10.3	12.0
6	.912	1.440	4.4	12.6	1.518	1.490	11.7	8.8
$NaHCO_3$ 3.4	—	—	7.9	14.6	—	—	2.24	2.20
" "	—	—	4.0	11.8	—	—	1.7	2.0

tate. Attempts are now being made to settle this point by adding fluoroacetate to kidney slices *in vitro*.

The infusion of acetate in the intact dog resulted in an increase in the oxygen consumption and PAH accumulation of the kid-

ney slices obtained from these dogs. This indicates that enough acetate or 2 carbon fragments had accumulated during the infusion period to stimulate the uptake of PAH to the same extent as 0.01M acetate added *in vitro* (compare Table I and III). The infusion of acetate further protected the kidney from NaFAc inhibition and the protection was roughly proportioned to the rate of acetate infusion.

The effect of fluoroacetate on oxygen consumption was relatively small compared to its effects on PAH accumulation. This is especially apparent in those experiments where no acetate was added. This indicates that only a small fraction of the total oxygen consumption is utilized for PAH transport. On the other hand fluoroacetate eliminated both the acetate induced increments in oxygen consumption and PAH uptake. This finding shows that acetate or the two carbon fragment is of primary importance in renal PAH transport(2).

Summary. Small doses of fluoroacetate mainly inhibit the ability of dog renal slices to respond to acetate stimulation as measured by PAH uptake and oxygen consumption. Larger doses of fluoroacetate produced inhibition of oxygen consumption and PAH uptake of renal slices in the absence of substrate. The effects on PAH uptake are more marked than are those on oxygen consumption.

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Stability of Serum Amylase.* (20598)

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Data available on the stability of pancreatic amylase in aqueous solution(1,2) indicate that highly purified preparations are quite stable but that crude preparations may lose their activity rapidly. However, a search of the literature disclosed a paucity of information regarding the stability of amylase in body tissues or fluids during storage. Scharles and Salter(3) state that aqueous extracts of the tumors of mouse sarcoma 180, preserved with toluene and kept in the refrigerator, retained their activity for several months. Fennel(4) states "urine or serum properly obtained and stored in the icebox at 4°C is trustworthy for 24, 48, or even 72 hours . . . the amylase content of serum remains remarkably constant in storage at 4°C; that of urine is not so stable." However, no quantitative data to support these statements were given in these papers and none was found elsewhere.

In clinical laboratories, it may be necessary to delay serum amylase determinations several days. Therefore, the question arises whether the amylase activity of stored sera remains constant.

Procedure. Samples of human blood were obtained from 14 normal, presumably healthy, staff members, technicians and secretaries of the University of Louisville School of Medicine. After clotting of the blood, the sera were immediately separated by centrifugation and analyzed for amylase activity by the method of Van Loon(5). Determinations of amylase activity were carried out immediately after separation of the sera (Day 1), one day

* This work was aided by a grant to the University of Louisville from the Kentucky State Medical Research Com.

TABLE I.
Amylase Activity of Human Sera, in 14 Subjects.

	Amylase activity of serum		
	Day 1	Day 2	Day 5
	87	82	77
	102	111	103
	86	89	86
	121	119	118
	61	74	86
	112	112	114
	64	66	81
	106	105	105
	105	104	103
	107	93	101
	76	85	93
	110	101	95
	39	44	48
	126	123	122
Avg	93.0	93.5	95.1
Stand. dev.		6.5	11.1
		9.2%	16.1%
Stand. error		1.8	3.0
		2.5%	4.3%
t		.28	.70
		.83%	1.30%

TABLE II.
Amylase Activity of Dog Serum in 5 Animals.

	Amylase activity of serum		
	Day 1	Day 2	Day 5
	1180	1190	1290
	2660	2370	2440
	900	1470	1450
	800	800	780
	940	940	940
Avg	1300	1350	1380

later (Day 2), and 4 days later (Day 5). The sera were stored in tightly stoppered test tubes in a refrigerator at 9-10°C between analyses. The results of these analyses are given in Table I. Sera obtained from 5 dogs were analyzed and treated in similar manner (Table II). Since the amylase content of dog serum is normally 10-20 times that of human serum, 1:100 dilutions of serum were used in these analyses instead of the standard 1:10 dilutions.

Results. The results shown in Table I were analyzed statistically using the method of paired variants. "Student's" t test was applied to the results using the hypothesis that no change in amylase activity occurred. Using the 5% value for t, which for 14 pairs of observations is 2.16, it was found that no change occurred. The t values in all cases were less than 2.16. Fiducial limits for Day 1-Day 2 were 0.5 ± 3.8 amylase units and 2.0 ± 5.3%; for Day 1-Day 5, 2.1 ± 6.5 amylase units and 5.5 ± 9.3%.

Discussion. In no case were the changes of such magnitude that they would lead to erroneous clinical diagnoses if serum amylase determinations were delayed. The increases in the amylase activities of the sera from P.M. and S.W. and from Dog 3 were significant but the mechanism of these increases is unknown. Work is now in progress testing the possibility of the formation of amylase activators or the presence of labile inhibitors. When the data on human sera were analyzed omitting the results for P.M. and S.W. there was no change in the general conclusion that the amylase activity of serum does not change during storage.

It is interesting that the serum amylase level of one subject, W.C., was definitely subnormal (normal range for Van Loon's method is 60-160) even though the subject showed no clinical symptoms of pancreatic insufficiency or hepatic disease.

Summary. The amylase activity of serum does not change significantly on a statistical basis when sera are stored at 9-10°C for 5 days. A few cases in which there were significant increases were noted, however.

The author wishes to express his appreciation to Dr. E. J. Van Loon for making the details of his serum amylase method available prior to its publication and for helpful comments and suggestions during the course of this work.

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Effect of Heat on the Agent of Homologous Serum Hepatitis.* (20599)

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The etiological agents of serum hepatitis and of infectious hepatitis are unusually resistant to chemical and physical methods of inactivation(1). The occurrence of numerous cases of homologous serum hepatitis following inoculation of certain lots of yellow fever vaccine to which human serum had been added has demonstrated that the agent of homologous serum hepatitis can survive heating at 56°C for 30 to 60 minutes(2,3). The human serum which was used in the manufacture of early lots of yellow fever vaccine was routinely "inactivated" by heating at 56°C for one hour. Actual practice, however, probably fell somewhat short of this(2). Oliphant, Gilliam, and Larson(4) showed that a sample of dried yellow fever vaccine from one of the incriminated lots was still capable of producing hepatitis after a further period of heating at 56°C for 30 minutes. Gellis, Neefe, Stokes *et al.* have demonstrated that a mixture consisting of 40 ml of normal serum albumin and 10 ml of proved icterogenic plasma failed to produce serum hepatitis when inoculated in 10 ml amounts into 5 volunteers after it had been heated at 60°C for 10 hours (5). The difference between the amounts of heat applied in heating at 56°C for one hour and at 60°C for 10 hours is considerable, and there is no indication of the factor of safety involved in heating under the latter conditions as is required in the manufacture of Normal Serum Albumin (Human)(6). Because of this, and the fact that heated plasma might be clinically useful were the amount of heat applied sufficient to inactivate the virus of serum hepatitis without serious damage to the proteins, it was decided to investigate the effect of heating at 60°C for 4 hours and for 2 hours by means of inoculation studies in human volunteers. Preliminary work had shown that heating at 60°C for 4 or more hours caused serious injury to the plasma as evidenced, among other things, by the occurrence of turbidity and the formation of a precipitate(7).

Effect of heating infected pool plasma at 60°C. The present study was part of an extensive program concerned with the safety of blood and blood products with respect to viral hepatitis, the general conduct of which is being reported elsewhere(8). Material from a single large pool of infected plasma, referred to as infected pool plasma, was used in all the studies on the safety of plasma and its derivatives conducted by our group.

Samples of infected pool plasma were subjected to controlled heating as follows: Four 10 ml bottles closed by means of sleeve stoppers and containing 7 ml of infected material were suspended in a water bath so that each bottle was completely immersed. The water was kept constantly agitated by means of a mechanical stirrer. Two bottles were removed after 2 hours of such heating, while the remaining 2 were removed after 4 hours. During this period of time, the temperature of the bath was maintained between 59.2 and 60.4°C. Two thermometers were used. A fifth similar bottle was kept at room temperature during the heating process. This served as a control. All bottles were shell-frozen by means of dry ice and alcohol immediately after heating and subsequently stored at —20°C until the time approached for administration to volunteer subjects. At this time the samples were transported to the institution where they were to be used in a Dewar

* This investigation was conducted in part under contract from the Office of the Surgeon General, Department of the Army, and under the sponsorship of the Commission on Liver Disease, Armed Forces Epidemiological Board.

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TABLE I. Effect of Heating Infected Pool Plasma at 60°C.

Duration of heating, hr	No. inoc.	Cases of hepatitis: With jaundice	Without jaundice	Total	Incubation period (days)
2	10	3	1*	4	70, 70,* 126, 147
4	10	3	2†	5	84,† 87, 88, 91,† 97
Not heated	5	1	1	2	77, 77*

* Case of hepatitis showing clinical signs and symptoms but no jaundice.
† Cases showing only abnormal laboratory findings.

flask under dry ice refrigeration. Some of the dry ice was still present when the flask was opened at the time of the inoculations. Two groups of 10 volunteers who had been carefully screened by a series of liver function tests were inoculated with heated material. One group received 1.0 ml of the plasma which had been heated for 2 hours at 60°C and the other 1.0 ml of that which had been heated for 4 hours at 60°C. Members of a third group of 5 men were each given 1.0 ml of the control material. All volunteers were kept under close observation for 6 months. The results are summarized in Table I.

It will be seen that 3 cases of hepatitis with jaundice and one showing clinical signs and symptoms of hepatitis but with no visible jaundice occurred in the group of 10 men inoculated with plasma which had been heated for 2 hours. Three cases of hepatitis with jaundice and 2 cases showing abnormal laboratory findings occurred in the group which received the plasma which had been heated for 4 hours. One case of hepatitis with jaundice and one case of hepatitis without jaundice occurred in the group which received the control material.

Experience with untreated infected pool plasma used as control material in this and other studies indicates that out of 55 subjects who received this material 22 (40%) developed hepatitis with jaundice, while 3 (5.5%) developed hepatitis without jaundice. Four additional subjects in these control groups showed laboratory findings suggestive of hepatitis.

Conclusions. Samples of plasma known to contain the agent of homologous serum hepatitis retained their ability to produce hepatitis even after being heated at 60°C for 4 hours.

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Absence of Hyperglycemic Effect of Glucagon in the Eviscerate Rat.* (20600)

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In these experiments, glucagon (a hyperglycemic principle occurring in extracts of pancreas) caused hyperglycemia in normal but not in eviscerated rats.

Methods. General details have been published(1,2). Male rats were eviscerated at 250 ± 2 g weight. All rats were studied under barbiturate anesthesia. The eviscerated rats were given solutions of glucose with and without insulin and antibiotics by continuous intravenous injection at the rate of 20 ml per 24 hours per rat. The normal rats received equal volumes of physiological saline. The glucose load is expressed as mg of glucose per 100 g of rat per hour (mg/100 g/h). When insulin (regular) was added, it was given at the rate of 4 units per 24 hours per rat. The antibiotic dosage was 10,000 units of penicillin and 5 mg of streptomycin per rat per 24 hours. A highly purified(3) preparation of glucagon was added to the glucose solution in some experiments, but in others, each dose was contained in 0.1 ml of physiological saline which was injected rapidly into the saphenous vein of the left hind leg. Glucose was determined (4) on tail blood. The temperature was 26.5 ± 0.5°C.

Experiments and results. In Exp. 1 the eviscerated rats received a glucose load of 64/100 g/h with insulin and the normal rats received only saline by continuous intravenous injection for periods of 90 minutes. Each rat received a separate injection of glucagon 30 minutes after the continuous injections were started. All doses of glucagon caused hyperglycemia in the normal rats but did not significantly affect the level of blood glucose in the eviscerated rats. The data are in Table I.

In Exp. 2 (Fig. 1) glucagon was given in the infusion fluid at a dose of 25 μg per rat per hour and an added dose of 25 μg contained in 0.1 ml of physiological saline was given separately at the time the continuous injections were started. Six pairs of eviscerated rats were given a glucose load of 64/100 g/h plus insulin for a period of 2 hours follow-

TABLE I. Effect of Glucagon upon Level of Blood Glucose in Eviscerate and Non-Eviscerate Rats. Insulin and glucose (64/100 g/h) to eviscerate rats. Glucagon administered separately. Individual values, mg %.

Dose μg glucagon	Non-eviscerate					Eviscerate				
		Min. post-injection					Min. post-injection			
	Initial	15	30	45	60	Initial	15	30	45	60
1	121	153	156							
10	126	153	195							
	127	142	186							
10	99	151	164	165	163	117	117	109	118	116
	102	147	186	181	174	120	118	87	123	118
	111	165	162	168	166	102	91	93	87	93
	108	153	165	183	181	138	145	148	159	165
	111	141	162	168	162	117	121	123	118	106
	108	144	172	162	138	96	75	69	69	66
25	115	177	192	186	156	117	99	106	111	120
	111	156	198	211	164	129	138	133	133	132
100	132	172	181	219	228	127	103	124	120	102
	126	187	189	207	192	129	133	135	165	168
250	120	171	199	216	213	96	103	85	87	90
	123	154	189	231	216	123	121	130	135	138

* We wish to express our thanks to Dr. W. R. Kirtley, Clinical Investigation Department, Lilly and Co., who supplied the glucagon for these studies.

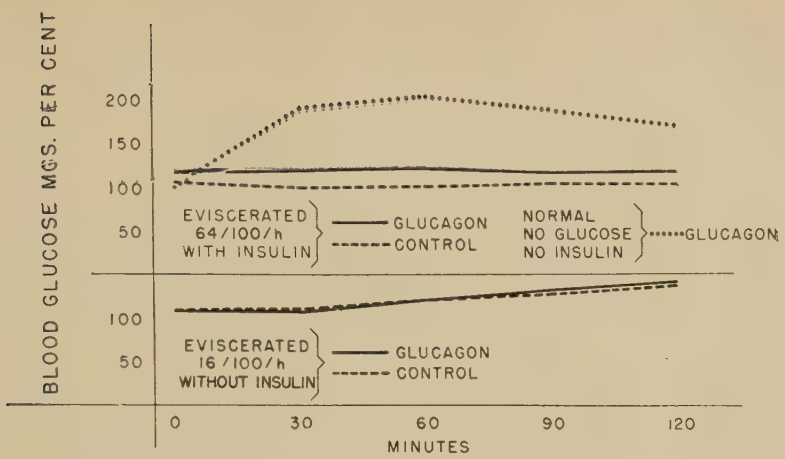

FIG. 1. Absence of hyperglycemic effect of glucagon in eviscerated rat; presence of hyperglycemic effect of glucagon in normal rat. Avg of 6 rats/group. Blood glucose determined at intervals of 30 min.

ing evisceration. Six pairs of eviscerated rats were given a glucose load of 16/100 g/h without insulin for 2 hours. One rat of each pair received glucagon whilst the other rat of each pair received added saline only. Six normal rats were given continuous intravenous injections of saline without glucose or insulin. The normal rats developed hyperglycemia following the injection of glucagon but the blood glucose of the eviscerated rats did not rise above that of their controls.

In Exp. 3, 19 pairs of eviscerated rats were each given a glucose load of 40/100 g/h with insulin and antibiotics for 24 hours. One rat of each pair received 500 μg of glucagon per 24 hours added to the infusion fluid. At the end of the 24 hours, the rats given glucagon had an average level of blood glucose of 105 $\pm$ 24 and the control rats, an average of 112 $\pm$ 8.5.

In order to test the stability of glucagon, a solution of 10 mg in 100 ml was kept at room temperature for 24 hours and then administered by continuous intravenous injection to 4 normal rats under barbiturate anesthesia for a period of 30 minutes. Each rat received 10 μg of glucagon. Hyperglycemia developed in each animal, the average value for blood glucose being 109 mg % at the beginning of injection and 183 mg % at the end of 30 minutes.

Discussion. It has been shown that hepatic glycogenolysis occurs following the injection of glucagon(5). The data of the present study fail to show any extra-hepatic effect of glucagon upon the tolerance of the rat for intravenously administered glucose but these observations do not exclude the possibility that glucagon has extra-hepatic effects upon metabolism.

Summary. Eviscerated rats were given continuous intravenous injections of glucose. The intravenous injection of glucagon failed to affect the level of blood glucose in eviscerated rats but did cause hyperglycemia in normal rats.

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Autoradiographic Arsenic Localization in Adult and Embryonic Epithelium and Connective Tissues.* (20601)

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It is well known that absorbed inorganic arsenic in mammals is localized in and excreted via the skin, nails and hair. However, biochemical, histochemical and isotope technics are in disagreement or leave doubt as to the exact tissue sites of predilection. As part of a pathologic study(1) which demonstrated cancers arising in human skin from epidermis, sweat and sebaceous glands, and probably also from hair follicles, an attempt was made to determine in animal tissues the localizations of radioactive arsenic, using autoradiography. Uptakes were tested in intact adult mice and in tissue cultures of embryonic mouse epithelium and chick fibroblasts.

Methods. The radioactive arsenic was received as a mixture of As^{73} and As^{74}. A single analysis showed 3.406 mg of arsenic per ml of the original solution in 0.059 N hydrochloric acid. Before injection a pH 6.4 phosphate buffer was added. The radioactivity level was redetermined before use. Mice of ABC strain, either sex, weighing 20 ± 5 g were used. Single injections were made:

Vol, ml	Buffer, ml	As^{73}, μc	As^{74}, μc	Inj. (vein)	Sacrificed
.2	.1	28.4	5.15	Jugular	1 hr
.3	.1	42.6	7.72	Caudal	20
.3	.1	42.6	7.11	"	72

There was insufficient radioactivity in mouse tissues from these experiments fixed by freezing-drying or in 10% formalin and embedded in paraffin to permit successful autoradiography.

On this account repeated doses were employed:

Vol, ml	Buffer, ml	As^{73}, μc	As^{74}, μc	Inj.	Interval
.5	.3	67.5	10.1	Intrap.	
.5	.3	67.5	10.1	"	1 hr later
.75	.2	101.25	15.0	"	22 hr after 2nd inj.

* This work was done under U. S. Atomic Energy Commission Contract AT (30-1)-901 with the New England Deaconess Hospital.

Sacrifice was carried out one hour later. The total dose was 236.25 μc of As^{73} and 35.2 of As^{74} in 1.75 ml of arsenic solution plus 0.8 ml of phosphate buffer. Total arsenic administered was 5.95 mg.

The mouse tissues were fixed by freezing-drying and embedded in paraffin(2). Sections were cut at 6-8 μ thick, and preparations were completed on the 5th day after sacrifice. Autoradiographs set up at this time were developed after 10 weeks of exposure.

Autoradiographs demonstrated arsenic in skin sharply and strikingly localized to epidermis, hair follicles, and other skin adnexa (Fig. 1, A and B). Dermal deposit was practically nil. In other tissues such as lung and kidney localization was evident chiefly in blood vessels. In the kidney a rich juxtamedullary cortical circulation was demonstrated, suggestive of a relative ischemia of the outer renal cortex (Fig. 2, A and B). This would be consistent with incipient shock(3).

Tissue cultures were carried out by the "flying cover-slip" technic(4), using a nutrient solution composed of 4 parts of human ascitic fluid, 1 part of chicken plasma, 5 parts of balanced salt solution, and 1 part of penicillin 1000 units/ml(5). Fibroblast cultures were obtained from 8-9 day old chick embryos and embryonic mouse epithelium from C_{57} strain fetuses. Cultures were grown at 38°C.

Radioactive or ordinary arsenic trichloride was added to separate fibroblastic and epithelial cultures, in dilutions of 1 part arsenic to 80000 parts of medium. Explants cultured were a) with radioactive arsenic, b) with ordinary arsenic trichloride and c) control with ordinary media. The radioactive solution was dilute enough to permit tissue growth without morphologic evidence of radiation damage.

After 6 days incubation the cultures were fixed in 10% formalin buffered to pH 7.0 and stained with hematoxylin and eosin, or used

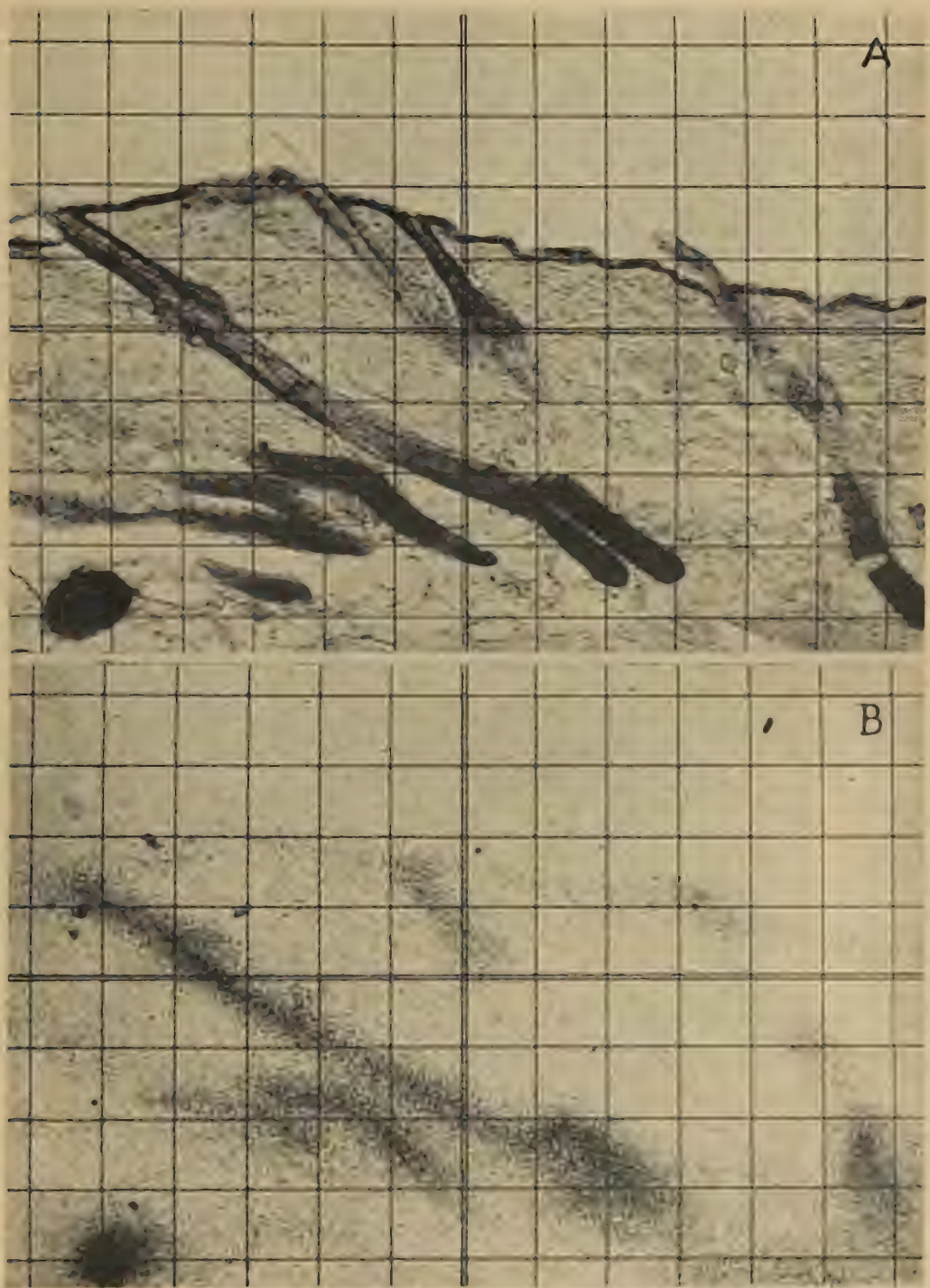

FIG. 1. a) Frozen-dried dry-mounted section of mouse skin, stained with hematoxylin and eosin after autoradiography. b) Autoradiograph from identical skin section, demonstrating arsenic radioactivity sharply localized to hair follicles, skin adnexa and epidermis. ×100.

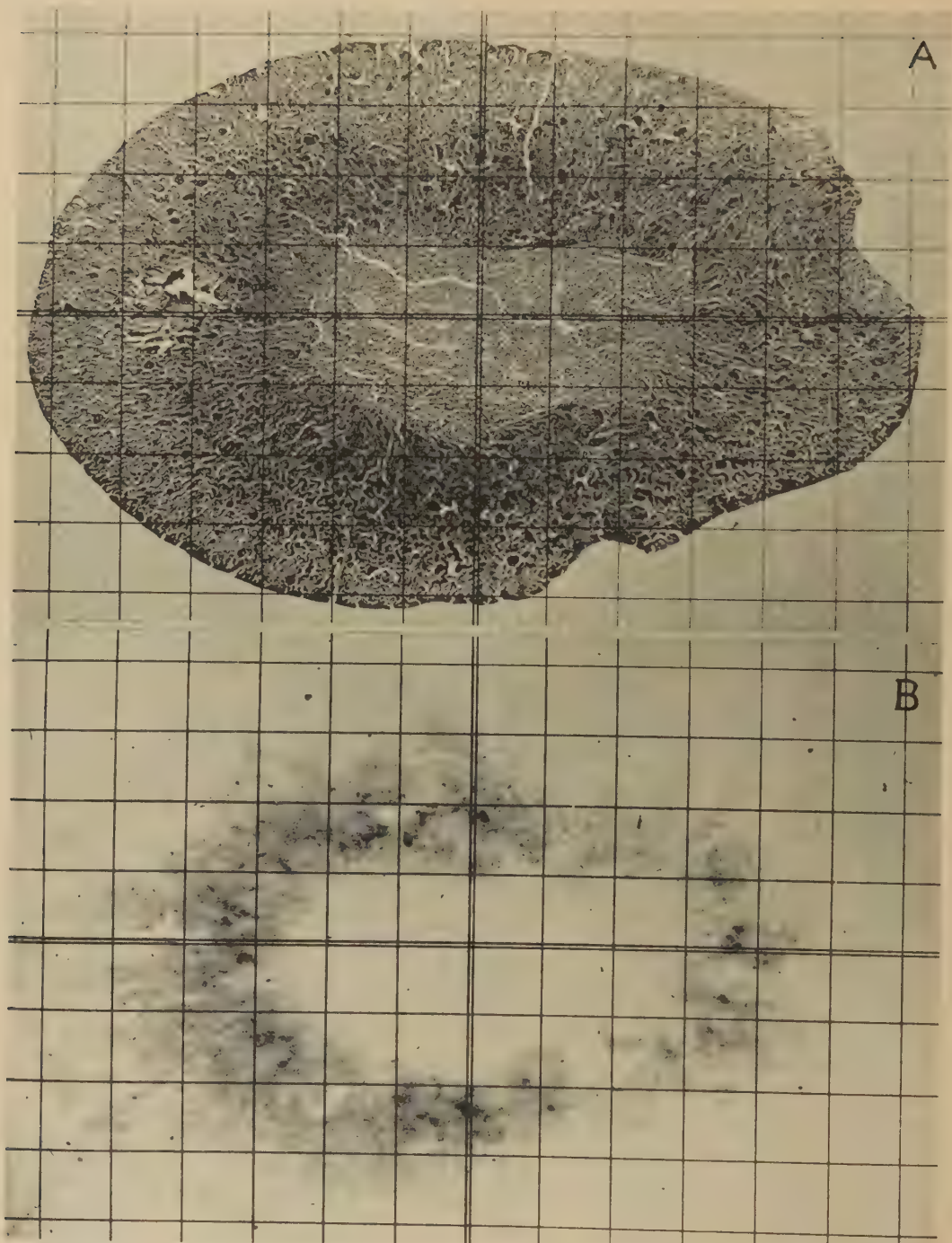

FIG. 2. a) Section of mouse kidney similarly prepared. Juxtamedullary cortical vascular congestion is present. b) Autoradiograph of identical section, showing intravascular arsenic radioactivity, following 3 injections during a 24 hr experiment. ×24.

for autoradiography, or both. Examination of both normal control cultures and those exposed to radioactive arsenic showed good histologic preservation and active proliferations at the edges composed of the respective epithelial or fibroblastic cellular components.

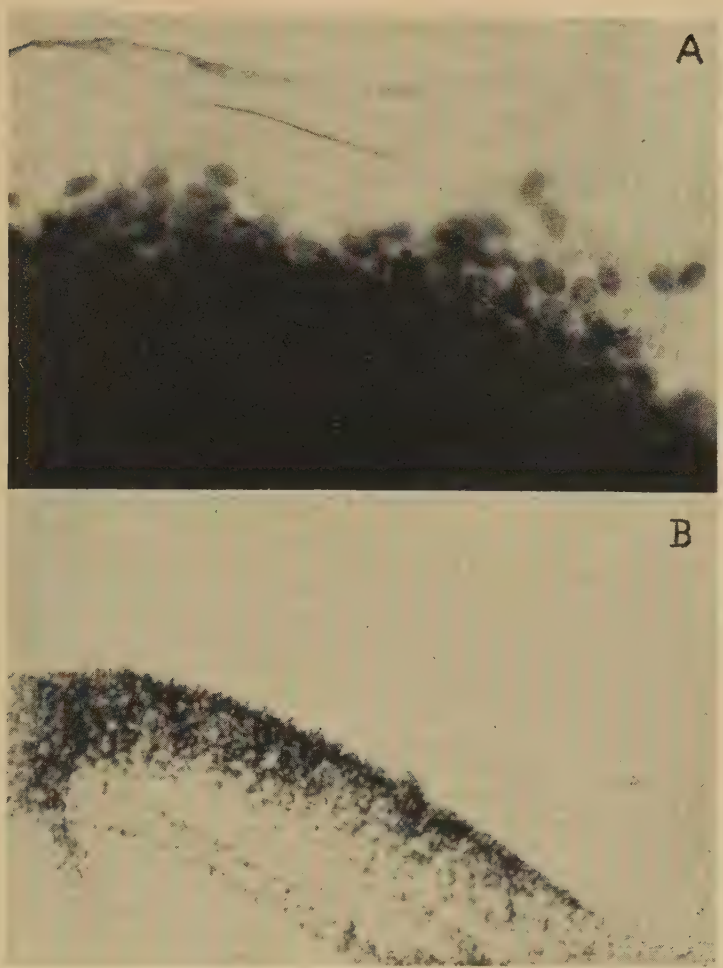

FIG. 3. Comparison of radioactive arsenic treated epithelial tissue culture margins a) stained as described, and b) as demonstrated by autoradiography. Fields are not identical. ×500.

Uptakes of radioactive arsenic were strong both in tissue cultures of embryonic epithelium (Fig. 3, A and B) and fibroblasts (Fig. 4, A and B), as demonstrated by autoradiography. The fibroblast culture uptake was slightly greater.

Controls exposed to 1:80000 dilutions of ordinary arsenic trichloride showed considerable necrosis of embryonic fibroblasts, but with some peripheral macrophage migration. Evidently the actual concentration of radioactive arsenic fell below the level of chemical toxicity. The technical difficulties of obtaining sufficient isotopic arsenic limited more extensive explorations.

Histochemical tests(6) for arsenic carried out on the same tissues were negative.

Comment. To our knowledge, use of radioactive inorganic arsenic in autoradiography has not been previously reported, although As^{74} tagged lewisite localization in skin has been studied(7). The autoradiographic results have suggested a contrast in the metabolism of arsenic in adult versus embryonic tissues. The epidermis and its adnexa were the specific sites of localization in the adult, while both embryonic fibroblasts and epithelium in tissue cultures absorbed arsenic with almost equal vigor. It would appear that the carcinogenic arsenic is actually contained within the types of cells where neoplasms later develop. Histochemical tests used at present thus fail accurately to localize the arsenic and therefore are not useful in such investigations

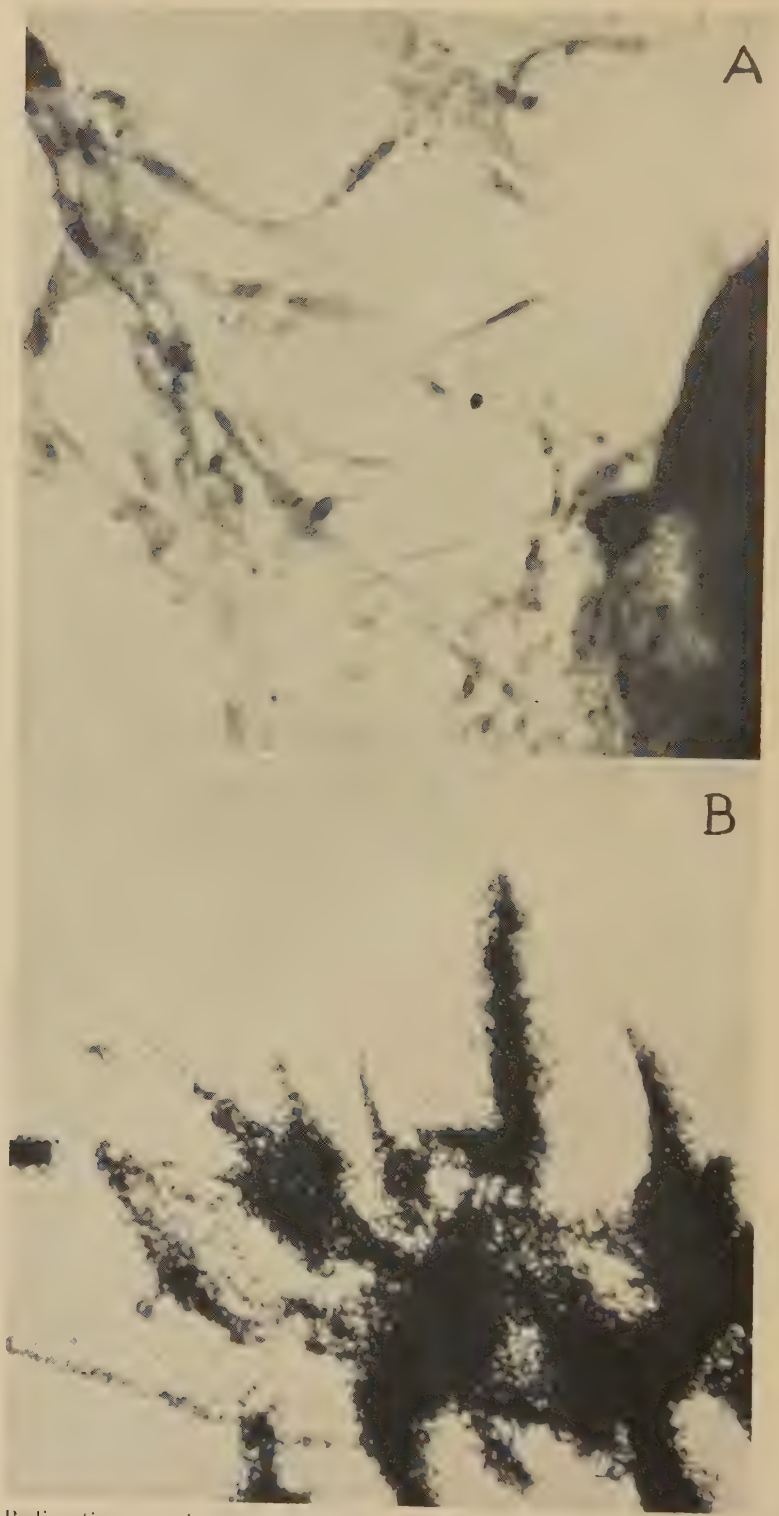

FIG. 4. Radioactive arsenic treated fibroblast tissue culture for comparison of margins a) stained as described b) shown by autoradiography. Fields are not identical. ×500.

of human tissues.

Summary. Autoradiography showed specific localization of As^{73} and As^{74} in adult mouse epidermis and skin adnexa. In tissue cultures embryonic mouse epidermis and chicken fibroblasts both absorbed the radioactive arsenic. Histochemical methods so far developed have evidently not accurately demonstrated the locations of arsenic in tissues.

Appreciation is expressed to Dr. Ralph W. McKee for the chemical analysis, to Miss Egilda DeAmicis for the assays of radioactivity, and to Dr. Margaret W. Holt and Miss Marie Sullivan for assistance with the autoradiography.

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Time-Response Effect of Cortisone upon Liver Glycogen in the Rat. (20602)

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The acute glycogenic effect of cortisone is known. In the present experiments cortisone acetate was administered to force-fed rats for periods up to 6 weeks. The peak level of liver glycogen was reached within 5 days and thereafter began to decline but remained at higher-than-normal levels during the times studied here.

Methods. Male rats of the Sprague-Dawley strain, having an initial weight of approximately 300 g, were force-fed a medium carbohydrate diet(1). All of the animals were force-fed for 14 days prior to beginning the injection of cortisone acetate. Cortisone acetate (microcrystalline suspension, Upjohn) was administered subcutaneously each morning. At the end of the injection period each rat was fasted for 24 hours and anesthetized with cyclopal; the liver was rapidly excised, weighed and dropped into hot KOH. Liver glycogen was determined by the use of anthrone(2).

Experiments and results. The cortisone dosage was 2, 5 and 10 mg/rat/day. At each of the 3 dosage levels, 6 to 8 rats were killed at intervals of 1, 2, 3, 5, 7, 10, 14, 21, 28 and 42 days. Liver glycogen was determined in 8 untreated rats. Rats given 10 mg of cortisone daily developed generalized infection between 4 and 6 weeks. Four of these rats died and the remaining 3 were sick and unsuitable for the determination of liver glycogen. The average values are summarized in Fig. 1. The increase was proportional to the dose of cortisone acetate. The peak level was reached by 5 days at each of the 3 dosage levels. Thereafter, there was a decline in the amount of liver glycogen but the time-response curves (Fig. 1) did not approach the values for untreated animals during the periods studied here. The values were also calculated as total glycogen per rat and as per cent glycogen in liver. Although these values did not correlate exactly with those shown in Fig. 1, the shape of the curves remained essentially the same. The concentration of glycogen in liver reached 9.26% for the group treated with 10 mg of cortisone acetate daily for 5 days.

Discussion. The decline in the time-response curve showing the effect of cortisone upon the level of liver glycogen may represent

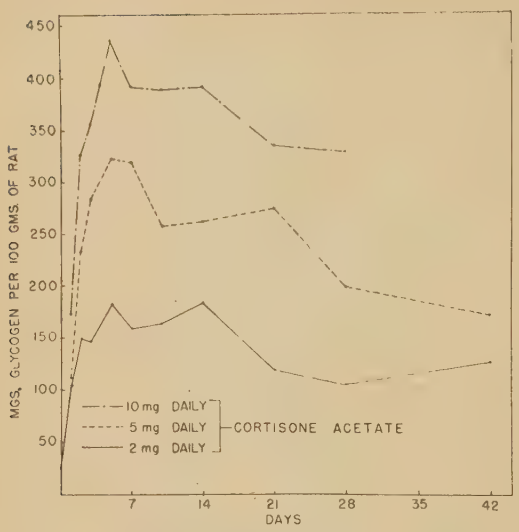

FIG. 1. Values for liver glycogen in rats treated with cortisone acetate. Each point in the curves represents avg of 6 to 8 rats. Expressed as mg of liver glycogen per 100 g of rat.

adaptation to hypercorticalism. There is a similar decline in the time-response curves showing the effect of high cortisone dosage upon urinary non-protein nitrogen and the glycosuria of rats having steroid diabetes induced by cortisone(3). This is not the only interpretation which can be made of the data. It was noted that the livers of these animals became progressively fatty especially at cortisone dosage of 5 and 10 mg daily. Baker *et al.* (4) studied similar animals having hypercorticalism induced by corticotropin and suggested that sufficient fat accumulated in the liver so that some of its functions might have been impaired. Partial impairment of hepatic participation in gluconeogenesis from protein might explain the reduction in the level of liver glycogen and also the decline in urinary nitrogen and glucose from their initial peak levels.

Summary. Male rats having an initial weight of approximately 300 g were given single daily injections of cortisone acetate in doses of 2, 5, and 10 mg/rat/day. At each of the 3 dosage levels 6 to 8 rats were killed at intervals ranging from 1 to 42 days and the amount of liver glycogen determined in each rat. The peak level was reached within 5 days and was followed by a significant decline towards, but not approaching, normal values.

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Single Dimension Chromatographic Separation of Thyroxin and Triiodothyronine.* (20603)

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The presence of the amino acid 3:5:3′ triiodothyronine in the plasma of normal and hyperthyroid subjects was recently demonstrated by Gross and Pitt-Rivers(1). These workers separated triiodothyronine from thyroxine by means of two dimentional chromatography. We have found this procedure time-consuming, and the spots produced tend to be diffuse and lack sharp definition. Described below is a method by which thyroxin‡ and triiodothyronine§ can be conveniently sepa-

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† Research Fellow of the Arthritis and Rheumatism Foundation.

‡ Kindly supplied by E. R. Squibb and Sons.

§ Kindly supplied by Smith, Kline and French Laboratories.

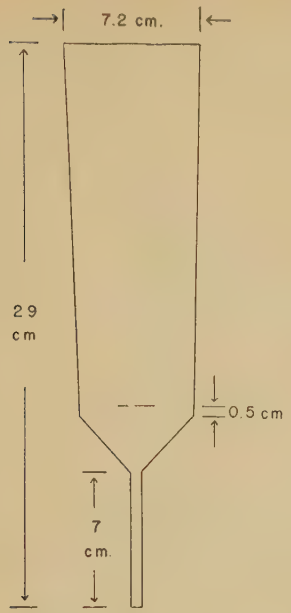

FIG. 1. Diagram of paper chromatogram.

rated by single dimension chromatography.

Method. An ascending type of chromatography was used. Whatman No. 3mm filter paper cut into a tapered form (Fig. 1), was employed(2). This has proved helpful in maintaining discrete spots; hence materially aids the separation of two amino acids with Rf values in the same range. A 4″ x 6″ x 14″ glass jar was used as the chromatographic chamber. The strips were suspended by stainless steel clips attached to brackets on the underside of the lucite lid. The tip of the paper wick was submerged 1-2 mm below the surface of the developing solvent. The jars were placed in a well insulated chamber to avoid variations in temperature. Several solvent systems were tried. In our hands butanol-primary amyl alcohol-ammonium hydroxide as employed by Robbins(3), and butanol-isoamyl alcohol-ammonium hydroxide as reported by

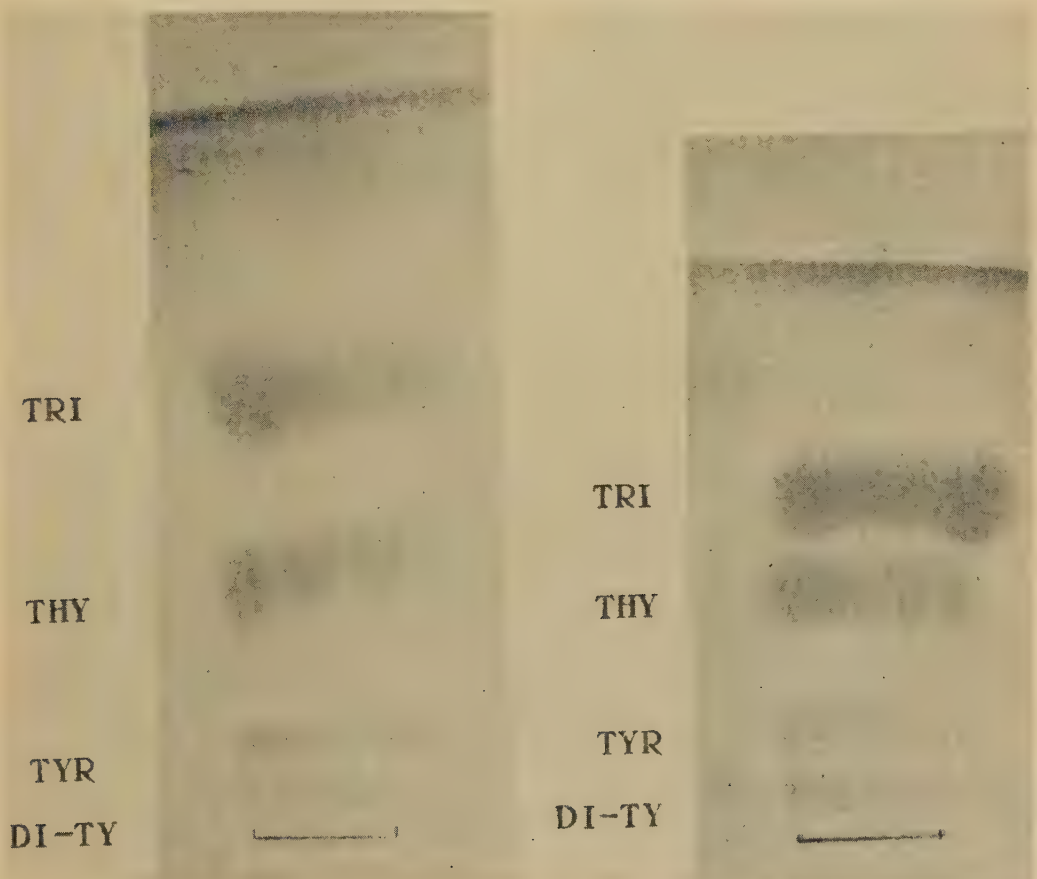

FIG. 2. Chromatograms of synthetic samples: *Left,* in butanol-dioxane-ammonia developer; *right,* in collidine-water developer. THY—thyroxin, TRI—3:5:3′-L-triiodothyronine, TYR tyrosine, DI-TY—diiodotyrosine.

Hird(4) gave less satisfactory separation of the amino acids than the following solvents. One consisted of 44 ml of water dissolved in 125 ml of 2-4-6 collidine. The other was a mixture of 80 ml of butanol, 20 ml of dioxane, and 100 ml of 2 N ammonium hydroxide. This mixture was shaken in a separatory funnel and the aqueous phase discarded. A small beaker of concentrated ammonium hydroxide was placed in the chamber containing the collidine-water solution to saturate the atmosphere with ammonia. The *amino acids* tyrosine, diiodotyrosine, diiodothyronine, triiodothyronine and thyroxin were dissolved in 0.5 N sodium hydroxide. Solutions containing 1 mg per ml of these amino acids were prepared. These were chromatographed singly and in combination. In the serum study I^{131} labeled triiodothyronine‖ and radioactive thyroxin‖ were added to serum at a concentration of 1 μg per ml. Two ml of this serum was extracted 3 times with twice the volume of butanol. Colorimetrically identifiable quantities of the amino acid were added and the extract was concentrated to 1 ml. The solution to be chromatographed was deposited on the paper in a horizontal line 2 cm long, 9 cm from the end of the wick. Care was taken to limit the

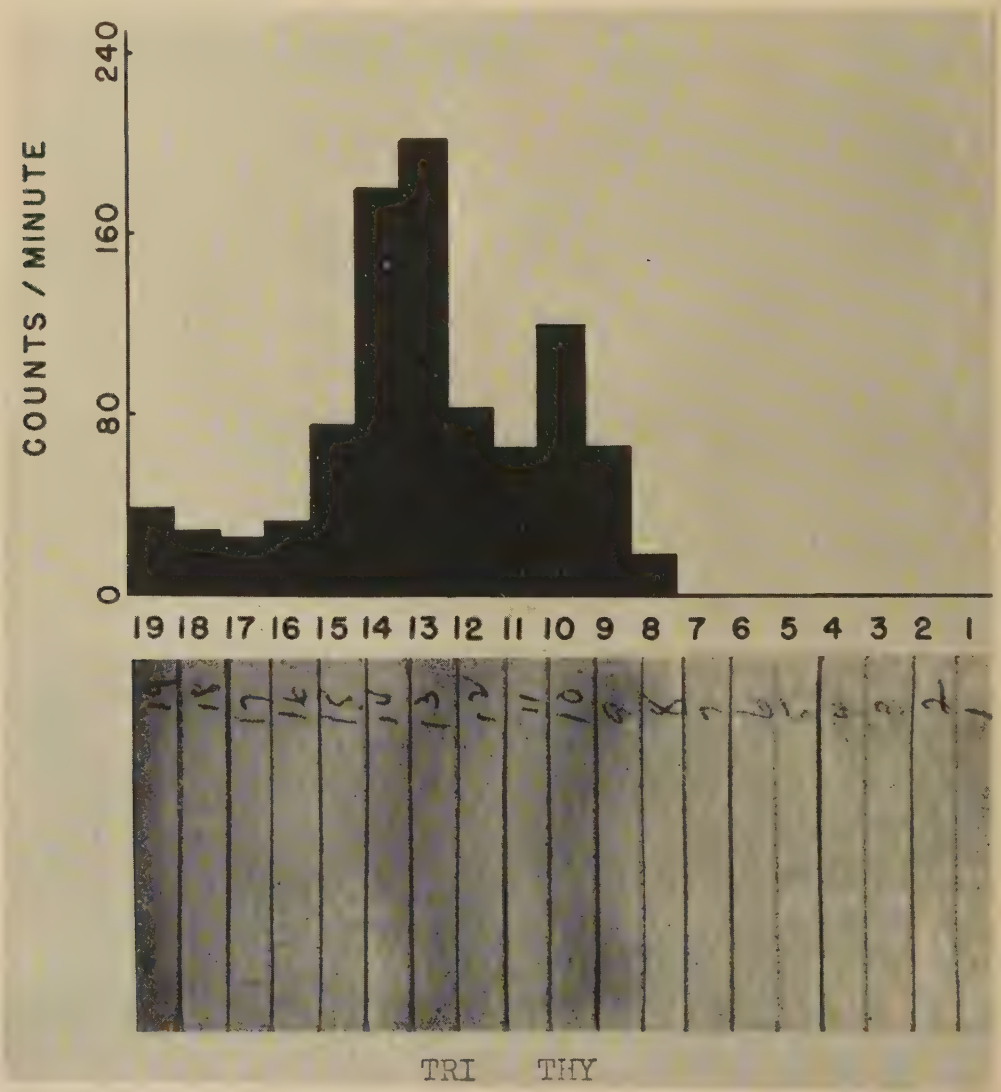

Fig. 3A

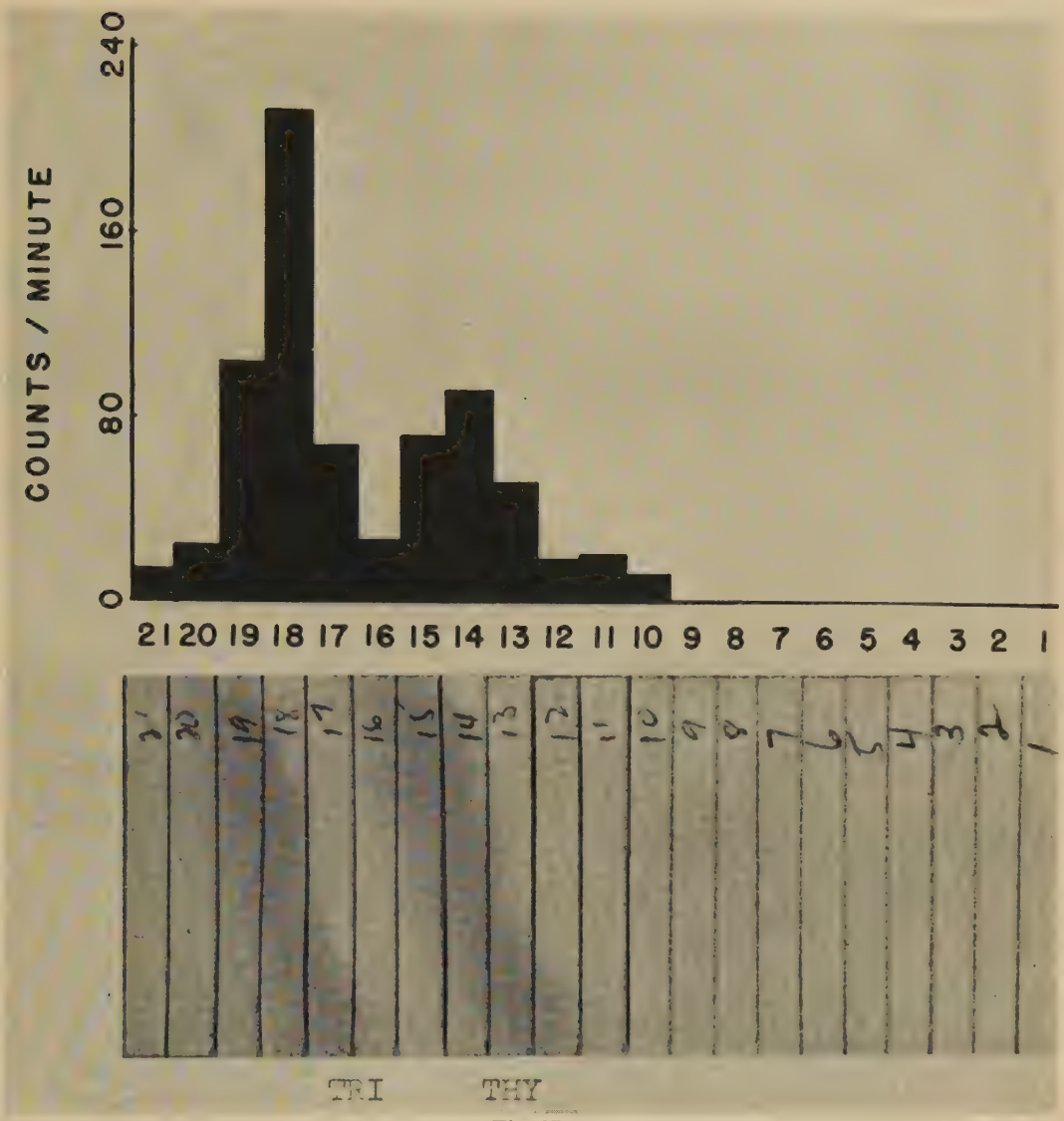

Fig. 3B

FIG. 3. Distribution of radioactivity on the chromatograms of butanol extracts of serum to which radiothyroxin and radiotriiodothyronine were added. A—collidine-water developer, B—butanol-dioxane-ammonia developer. Position of the amino acid is indicated by legend, THY—thyroxin, TRI—triiodothyronine.

width of the line to 2-3 mm. If the strip is warmed during the application of the solution, 50 microliters can be conveniently applied on this area. Eighteen hours were allowed for development. At the end of this period the strips were removed and dried at approximately 80°C. They were then sprayed with a 2.5% sodium carbonate solution and again dried. Finally, they were sprayed with a diazo reagent prepared according to Koessler and Hanke(5).¶ Approximately 50 μg of each amino acid are required for adequate intensity of color using this reaction.

In the serum studies in which the radioactive amino acids were used the paper strips were cut transversely into segments 5 mm in

‖ Obtained from Abbott Laboratories, Oak Ridge, Tenn.

TABLE I. Rf Values of Several Iodine Containing Amino Acids, Tyrosine and Iodide in Butanol-Dioxane-Ammonia and Collidine Solvents at 18 Hr at Room Temperature.

	BDA	Collidine
Thyroxin	.37	.45
3:5:3'-L-triiodothyronine	.64	.60
3:5-Diiodothyronine	.52	
Diiodotyrosine	.05	.08
Tyrosine	.12	.25
Iodide	.33	1.0

width and the radioactivity of the segments determined in an end window GM counter.

Results. Typical chromatograms of pure amino acid solutions are seen in Fig. 2 which demonstrates separation of thyroxin and triiodothyronine in both solvent systems. Thyroxin, triiodothyronine and diiodothyronine appear as pink lines with the diazo reagent while tyrosine and diiodotyrosine produce an orange color. Table I lists the Rf values observed in each solvent system.

¶ Prepared as follows: To a 50 ml flask immersed in an ice bath add (1) 1.5 ml of a solution containing 9 g sulfanilic acid and 90 ml of concentrated HCl per liter and (2) 1.5 ml of a 5% sodium nitrite solution. Allow to stand 5 min. and add an additional 6 ml of sodium nitrite solution. After 5 more min. dilute to volume with water. This reagent may be used for several days if refrigerated.

Fig. 3 shows the distribution of radioactivity on the chromatograms of butanol extracts of serum to which radioactive thyroxin and radioactive triiodothyronine have been added. Coincidence of radioactivity with the amino acid spot is shown by the accompanying bar graph.

Summary. A method is described by which iodine-containing amino acids, including thyroxin and triiodothyronine can be adequately separated by single dimension chromatography. Physiological quantities of labelled thyroxin and triiodothyronine have been added to serum, extracted with butanol and separated by this method.

The authors wish to acknowledge the invaluable assistance of Miss Sue Ames, Medical Technician.

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Lack of Growth-Promoting Potency and of Toxicity of Glucagon (Hyperglycemic-Glycogenolytic Factor) in Hypophysectomized Rats. (20604)

I. I. GESCHWIND AND A. STAUB. (Introduced by Choh Hao Li.)

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The hypothesis that pituitary growth hormone stimulates the release of glucagon from the islet cells of the pancreas(1,2) has recently received experimental support from several laboratories(3-5). Elrick(6) has attempted to adduce further evidence for this claim by determining whether the growth promoting action of growth hormone is mediated through glucagon. A slight increase in the width of the proximal epiphyseal cartilage of the tibia was reported following glucagon administration to hypophysectomized rats. Since these results seem to add further weight to the aforementioned hypothesis, the experiments were deemed worthy of repetition with a highly purified glucagon preparation.

Experimental. The glucagon preparation (lot No. 208-108 B-234) employed in this study was a highly active preparation in which insulin had been destroyed by treatment with cysteine. Administration of 0.5 μg/kg intravenously into cats caused a 30 mg % rise in

TABLE I. Effect of Glucagon on Tibial Cartilage Width of Hypophysectomized Rat.

Exp. group	No. of animals	Daily dose (μg)	Cartilage disc width ($\mu \pm$ S.E.)
Control	12	0	147 ± 1.6
Glucagon*	6	5	147 ± 2.5
" *	7	50	146 ± 2.2
" †	7	100	155 ± 2.3
" †	5	250	156 ± .9

* Solution for inj. for whole experiment prepared in advance.

† Sol. for inj. prepared daily from freshly weighed powder.

blood sugar. Insulin assay indicated that the preparation was virtually free of hypoglycemic activity. The insulin employed was a crystalline preparation (lot No. 538, 155) with a potency of 26 units per mg. Growth hormone assays were performed in female rats of the Long-Evans strain, hypophysectomized at 28 days of age. Injections were begun 2 weeks post-operatively, and were continued for 4 days. All injections were of 0.5 ml volume and were given intraperitoneally, with the exception of one experiment in which the glucagon was suspended in a peanut oil-beeswax mixture (7), and injected subcutaneously in a daily dose of 0.1 ml. The animals were sacrificed 24 hours after the final injection and the tibial cartilage widths were determined after staining with silver nitrate according to the method of Greenspan *et al.*(8).

Results and discussion. The results presented in Table I demonstrate that doses of glucagon as great as 250 μg per day for 4 days are completely incapable of causing any increase in the disc width; that is, glucagon shows no evidence of any growth promoting action by this test. In the 3 experiments reported by Elrick(6), 3 different preparations of glucagon in doses of from 10 to 100 μg per day caused increases in the tibia widths. However, in 2 of the 3 experiments no dose-response relationship was observed, and in none of the experiments was a cartilage width found which was outside the range of non-specific response(8). It should be borne in mind, additionally, that none of the preparations of glucagon used in these earlier experiments was as pure as the one herein employed. This is evident from the increased hyperglycemic activity of our preparation, its freedom from insulin contamination, and its complete lack of toxicity in the doses employed. The last point is of special interest, since it has been claimed that doses of glucagon greater than 25 μg per day are toxic to 20 to 60% of the animals during a 4-day test(6). The present experiments, employing much higher doses of glucagon than those employed by Elrick(6), demonstrated that the toxicity is neither an inherent property of the glucagon, nor is it a consequence of its physiological action.

The possibility existed that the cruder preparations previously employed(6) may have contained contaminants which delayed the absorption of the injected glucagon, and hence increased its duration of action. In order to test this alternative, glucagon was administered subcutaneously in beeswax for 4 days, but without any apparent toxicity. Comparison of the control data and the experimental data in line 2 of Table II, demonstrate that the administration of glucagon in beeswax is of no assistance in promoting a growth response.

A final remaining possibility was that both the increase in tibia cartilage width and the toxicity previously reported(6) were caused by contamination of the earlier preparations of glucagon with insulin. Thus Salter and Best(9,10) have reported that injection of the hypophysectomized rat with protamine zinc insulin results in a stimulation of body and skeletal growth and that by the 15th day of injection the tibia cartilage width is twice the control value. On the other hand, the extreme

TABLE II. Effects of Glucagon in Beeswax, of Insulin, and of Glucagon in Combination with Insulin on Tibial Cartilage Width of Hypophysectomized Rat.

Exp. group	No. of animals	Daily dose (μg)	Cartilage disc width ($\mu \pm$ S.E.)
Control	8	0	170 ± 4.7
Glucagon*	6	100	171 ± 1.8
Insulin	5	.8†	172 ± 2.3
Insulin + glucagon	4	.8† 100	170 ± 5.0

* Daily subcut. inj. of 0.1 cc beeswax-peanut oil suspension for 4 days.

† Equivalent to .02 unit.

sensitivity of the hypophysectomized animal to regular insulin is well known. This possibility was investigated in 2 groups of animals, one of which was injected intraperitoneally with 0.02 unit of insulin daily for 4 days, whereas the second group received the same dose of insulin together with 100 μg of glucagon per day. The results of these experiments given in lines 3 and 4 of Table II, demonstrate complete lack of growth activity on the part of insulin alone in this dosage, or of insulin together with glucagon. Insofar as toxicity is concerned, one animal from each of these 2 groups died after the first injection, and all the other animals appeared in poor condition. The practice was therefore instituted of injecting one ml of a 20% glucose solution intraperitoneally one-half hour after the hormone injection. Under this regime, only one animal, from the combined hormone group, died in the following 3 days, and the general appearance of all animals appeared greatly improved. Although the desire to obtain more information about toxicity had to be relinquished in favor of obtaining a sufficient number of surviving animals for growth assay, it appeared, nonetheless, that insulin, at 0.02 unit per day, could be quite toxic to the hypophysectomized animal, and thus could possibly explain the toxicity of cruder glucagon preparations.

Summary. A highly purified preparation of glucagon in doses as high as 0.25 mg/day, has no effect on the width of the epiphyseal cartilage of the hypophysectomized rat as determined by the tibia test for growth hormone. Such a preparation possesses no toxicity when injected into these test animals. Possible reasons for the previously claimed growth promoting activity and toxicity of glucagon fractions are discussed.

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Effects of Interfering Virus and RDE upon Development of Toxic Reactions.* (20605)

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The occurrence of toxic reactions following intracerebral, intraperitoneal or intravenous injection of influenza viruses into certain species of animals was first described by Henle and Henle(1,2,3) and subsequently confirmed and elaborated upon by various other workers (4-7). Despite the approximately 9 years which have elapsed since the first description of the phenomenon, however, little is known about the nature of the toxins or the mechanisms of their action. Earlier reports(1-5) based on passage and cultivation experiments have shown that viral multiplication was not an essential factor in the induction of toxic episodes. These findings might possibly pose a question concerning the necessity for intimate contact between virus and susceptible cells in animals which exhibit toxic symptoms.

* This work was aided by a grant from the Research Committee of the University of California.

It seemed of interest, therefore, to determine the effects of prior injections of interfering virus and receptor-destroying enzyme upon the development of toxic reactions. The present paper describes the results of these studies.

Materials and methods. Virus. The strain of virus used in these studies was an egg-adapted strain of influenza A (PR8). Virus was prepared by suballantoic inoculation of 10- or 11-day embryonated eggs with 0.05 ml of a 10^{-5} or 10^{-6} dilution of virus in beef-heart infusion broth. Inoculated eggs were incubated at 36°C for 40-48 hours. After overnight chilling in the refrigerator, the allantoic fluids were harvested, pooled, and stored in ampoules in a dry ice cabinet. This was used as a source of toxic virus. *Preparation of interfering virus.* Virus prepared in the above manner was concentrated by the method of red cell adsorption and elution. A final concentration of 5% chicken red cells was added to virus; after 45 minutes in an ice water bath, the supernatant fluid was removed and the agglutinated cells resuspended in a small volume of 0.2 M phosphate buffer, pH 7.4. Elution was carried out in a 37°C water bath for 4 hours. The red cells were sedimented by light centrifugation and the supernatant fluid removed and adjusted to an hemagglutinin titer of 2560 (titer expressed as reciprocal of dilution of virus in last tube showing complete agglutination when read by pattern method). The viral concentrate was then exposed to 5×10^{-3} M sulfur mustard for 3 hours at room temperature. Virus inactivated in this manner gave rise to no hemagglutinins in each of 6 eggs inoculated allantoically with 0.05 ml of undiluted material or with 0.05 ml of serial 10-fold dilutions of virus. As reported previously, such preparations were markedly interfering(8). *Receptor-destroying enzyme. Vibrio cholerae* (Inaba), Culture 35A3 (National Institute of Health, Bethesda, Md.) was grown in beef-heart infusion broth for 18 hours at 37°C. The culture was passed through a Berkefeld N filter and the filtrate used as a source of RDE. The activity of this preparation was adequate to destroy all hemagglutinin-inhibitors in mouse brain extracts after 1 hour at 37°C. *Experiments with interfering virus.* Injection of inactivated influenza A (PR8) virus for determination of its effect upon development of toxic symptoms consisted of 0.03 ml of undiluted interfering suspension introduced into the right cerebral hemisphere of 3-week-old mice. (Swiss, Webster strain, random sex) under light ether anesthesia. This was followed 2 hours later by inoculation of 0.03 ml of active (toxic) influenza A virus into the same general area. Control mice received normal allantoic fluid in the first injection instead of interfering virus. All mice were observed daily for a period of 7 days. Convulsions upon spinning of animals or deaths occurring after 24 hours were used as criteria for virus-induced toxic reactions. *Experiments with RDE.* Intracerebral inoculation of RDE and toxic virus in mice and their subsequent observation followed the same general procedure described in the preceding section. Control animals were given broth instead of RDE in the first injection.

Results. Effect of prior injections of inactivated virus or RDE upon development of toxic reactions. The results of toxicity tests in mice receiving prior inoculations of either homologous interfering virus or RDE are shown in Table I. The data of this table represent the combined results of 2 to 3 separate experiments.

The suspension of active virus used in these experiments was toxic to slightly more than 50% of experimental animals when 0.03 ml of a 1:2 dilution was inoculated (Animal Group 3). The data for Animal Group 1 indicate that intracerebral inoculation of 0.03 ml of inactivated interfering virus 2 hours before administration of a similar volume of undiluted toxic virus resulted in a marked reduction in the incidence of toxic reactions. It may be seen that the ratio of toxic reactions in mice receiving prior injections of inactivated virus and in control animals (Group 2) is approximately 1:2.5. An even greater reduction in the number of toxic reactions was evident when 0.03 ml of RDE was administered prior to intracerebral inoculation of toxic virus. Comparison of the data shown for animal groups 4 and 5 reveals that the ratio of toxic reactions in the two groups is about 1:7. The results shown for Animal Group 6 emphasized the necessity for administration of RDE before virus if effective sup-

TABLE I. Effects of Prior Injections of Interfering Virus and RDE upon Development of Toxic Symptoms.

Animal group	Nature of 1st inoc.	Nature of 2nd inoc.	Toxicity results*	% positive reactions
1	Inactivated PR8	Undil. active PR8	9/24	37.5
2	Allantoic fluid	" " "	23/24	95.8
3	" "	½ dil. active PR8	13/24	54.2
4	RDE	Undil. active PR8	4/32	12.5
5	Broth	" " "	20/23	86.9
6	Undil. active PR8	RDE	22/23	95.7
7	RDE	Undil. active PR8†	24/24	100.

Time between inoc. = 2 hr. .
* No. of animals dead or showing convulsions/total No. of animals in test.
† Second inj. consisted of 1 ml of virus intrav. (All other inj. intracer.)

pression of toxic reactions is to be established. *Vibrio cholerae* filtrates given 2 hours after active virus failed to influence the subsequent course of events in mouse brain.

It is apparent from the data shown in Animal Group 7 that intracerebral inoculation of 0.03 ml of RDE 2 hours earlier failed to prevent the development of toxic symptoms upon intravenous injection of 1 ml of active influenza A virus. The action following intracerebral injection of RDE was apparently a local effect, presumably because the amount of RDE introduced into the host was so small. However, the activity of RDE within the mouse brain was not a unilateral one confined to the side of inoculation, for other tests (not shown) employing injections of RDE and virus into opposite hemispheres led to an equally effective suppression of toxic reactions, provided the receptor-destroying enzyme was given prior to virus.

Discussion and summary. The results reported in this paper have shown that prior injections of inactivated interfering virus or RDE into mouse brain resulted in a marked reduction in the incidence of toxic episodes usually associated with intracerebral administrations of concentrated suspensions of influenza viruses. Intracerebral injections of RDE failed to prevent toxic manifestations, however, when virus was introduced by an intravenous route. The somewhat greater efficiency of RDE in suppression of toxic manifestations, as compared with inactivated virus, may possibly be a reflection of its position in the receptor gradient. The fact that both inactivated interfering virus and RDE proved effective in protection of animals against toxic doses of virus introduced into the central nervous system might appear to suggest that intimate contact between virus and susceptible cell was essential for development of toxic reactions. If union of virus and cell is indispensable, it is apparently a fairly rapid process, for injection of RDE two hours after toxic virus failed to modify the incidence of toxic reactions.

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Comparison of Quantity of Egg and Mouse-Adapted Influenza Viruses Required to Infect Each Host. (20606)

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Certain characteristics of strains of influenza viruses, adapted to the chick embryo and to the mouse lung, have been considered to provide an inherent basis for differences in their biological behavior. Properties have been ascribed to mouse-adapted agents, that distinguish them from their relatives which, although "unadapted," can nevertheless multiply extensively in the mouse lung. Mouse-adapted strains propagating in the mouse lung have been reported to possess: 1) a more rapid multiplication rate(1); 2) a shorter "lag" phase of the growth cycle(2); 3) the capacity to synthesize more virus(2); and 4) the ability to destroy the inhibitor of hemagglutination in mouse lung(3). In addition, differences have been demonstrated in strains of viruses in their capacity to produce cell damage(4) and the effect of this injury on subsequent viral multiplication has been studied(4). In every instance the comparable quantity of each agent employed was determined solely by infectivity titrations in chick embryos whereas the properties of the influenza viruses were investigated in the mouse. In order to interpret these studies adequately and to carry out further experiments comparing influenza viruses it was important to test the validity of the assumption that comparable quantities of 2 strains measured in the chick embryo would maintain the same quantitative relationship in a second host, *i.e.* the mouse. Studies by Knight indicated that with the mouse-adapted PR8 strain of influenza A virus, mouse-passage preparations were about 100 times as infectious for mice as egg-passage preparations of this agent(5). It seemed possible, therefore, that the relative quantity of adapted virus necessary to infect the mouse would be considerably less than the concentration of unadapted agent essential to initiate viral multiplication. If so, this would provide another differentiating property of mouse-adapted influenza viruses. Moreover, it appeared that the quantitative capacity to infect a given host might vary from strain to strain, mouse or egg adapted. The results of a study of the relative quantity of mouse adapted and unadapted influenza viruses essential to infect the chick embryo allantoic membrane and the mouse lung are here described.

Materials and methods. Viruses. A number of different chick embryo- and mouse-adapted strains of influenza A virus and the mouse-adapted Lee strain of influenza B virus were employed. In Table I are indicated the source, animal passage history and whether or not they are adapted to the mouse lung. The strain names of these agents will be used for reference in this paper. Viruses were cultivated by inoculation of 0.2 ml of a 10^{-4} dilution of each into the allantoic sac of 10-11 day old chick embryos(6). Infected allantoic fluids were stored in sealed glass ampules at —70°C in a cabinet which contained solid CO_2. *Animals.* Three to 4 week old albino Swiss mice, CFW strain, were obtained from Carworth Farms for mouse infectivity titrations. Fertile white eggs received from a local dealer were incubated at 39°C for 10 to 11 days and then employed for *in ovo* infectivity titrations. *Viral infectivity titrations.* Dilutions of virus were prepared in 0.5 $\log_{10}$ increments in tryptose phosphate broth containing 500 units of penicillin and 0.5 mg of streptomycin per ml. Dilutions were kept in an ice-water bath, and each inoculated as quickly as possible into mice and embryonated eggs. *In ovo titrations.* Each dilution was inoculated intra-allantoically in 0.1 ml volumes into groups of 4 chick embryos. Inocu-

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TABLE I. Descriptive Data Concerning the Strains of Influenza A and B Viruses Employed.

Virus	Isolated by	Passage history‡	Mouse adapted
Influenza A—PR8	Francis	Unknown§ E1	Yes
—CAM*	Anderson	E210	No
—WRU 51-51	Jordan	E8	"
— " " "	"	E5; M13; E1	Yes
—WRU 52-51	"	E4	No
— " 53-51	"	E3	"
—Rhodes†	Francis	F3; E9	"
— " †	"	F4; M127; E1	Yes
Influenza B—Lee	"	Unknown§ E1	"

* Obtained from Dr. John Y. Sugg, Cornell University Medical College.
† Obtained from Dr. Fred M. Davenport, School of Public Health, University of Michigan.
‡ E = No. of chick embryo passages; M = No. of mouse passages; F = No. of ferret passages.
§ Isolated in ferrets; followed by numerous mouse and chick embryo passages.

lated eggs were incubated for 44-48 hours at 35°C, followed by 16-18 hours at 4°C. The presence of virus in the allantoic fluid of each egg was determined by the hemagglutination reaction(7): to undiluted and 1:10 dilution of each fluid was added an equal volume of 1% chicken erythrocytes and the pattern of RBC was observed after 60 minutes at room temperature. *Mouse infectivity titrations.* A combination mouse-egg infectivity technic was employed in that the presence of unadapted influenza viruses in mouse lung cannot be demonstrated directly. Mice were inoculated intranasally under light ether anesthesia with 0.05 ml of the diluted material to be tested; groups of 4 mice per dilution were employed. Mice were killed 3 days after inoculation, and the lungs removed asceptically from each group starting with mice which had received the highest dilution. A 10% suspension of lung from each mouse was prepared individually by grinding in a mortar and pestle with 1.5 ml of tryptose phosphate broth which contained penicillin and streptomycin. Suspensions were centrifuged at 2000 rpm for 2 minutes, and each supernate was inoculated into the allantoic sac of each of 3 embryonated eggs. The inoculated eggs were incubated for 44-48 hours at 35°C and the presence of virus determined exactly as described under *in ovo* infectivity titrations. A mouse was considered to be infected if 1 or more of the 3 allantoic fluids had virus present. The results of titrations were expressed as 50% egg ($E.I._{50}$) or mouse ($M.I._{50}$) infectivity titers (8).

Experimental. The infectivity titer of an organism may be defined as the smallest number of particles of the agent which can infect a given host. This quantitative expression then indicates the relative amount of the organism in one infectious unit. By means of titrations in 2 hosts with the same dilutions of a single virus, the comparative quantity of the agent required to initiate infection in each host may be determined. Or expressed somewhat differently, one may estimate the relative susceptibility of each host to the agent. Experiments were designed to determine with chick embryo and mouse-adapted influenza viruses the relative quantity of virus in a single infectious unit for each of the 2 hosts.

The results of an experiment carried out with mouse-adapted and chick embryo-adapted lines of the WRU 51-51 strain of influenza A virus are presented in detail in Table II. The egg infectivity titer was 2.5 log units higher than the mouse infectivity titer when the strain which had been passed only in embryonated eggs was employed. In sharp contrast to this finding were the data obtained with the WRU 51-51 virus which had been adapted to the mouse lung by serial passages. With this strain the dilution of virus which infected the mouse was almost identical with that essential to initiate viral propagation in the allantoic sac of the chick embryo. These findings suggest that to infect a mouse approximately 100 times as much egg-adapted as mouse-adapted influenza virus is required.

To determine if the quantity of virus in an

TABLE II. Results of Simultaneous Egg and Mouse-Egg Infectivity Titrations Carried Out with Mouse-Adapted and Egg-Adapted Strains of WRU 51-51 Influenza A Viruses.

Virus titered	Dilution inoc.	Egg infectivity titration		Mouse-egg infectivity titration						
		Score†	Titer, $E.I._{50}$‡	Egg infectivity score† 1*	2	3	4	Mouse lung score	Titer, $M.I._{50}$§	Ratio $E.I._{50}/M.I._{50}$, log
WRU 51-51, egg-adapted	$10^{-5.0}$			3/3	3/3	3/3	3/3	4/4		
	$10^{-5.5}$			0/3	"	2/3	0/3	2/4	$10^{-5.6}$	
	$10^{-6.0}$			3/3	0/3	0/3	"	1/4		2.5
	$10^{-6.5}$	4/4		0/3	"	"	"	0/4		
	$10^{-7.0}$	"		"	"	"	"	"		
	$10^{-7.5}$	"		"	"	"	"	"		
	$10^{-8.0}$	2/3	$10^{-8.1}$							
	$10^{-8.5}$	0/4								
WRU 51-51, mouse-adapted	$10^{-5.0}$			3/3	3/3	3/3	3/3	4/4		
	$10^{-5.5}$	4/4		"	"	"	"	"		
	$10^{-6.0}$	"		"	"	"	2/3	"		
	$10^{-6.5}$	"	$10^{-6.8}$	"	"	0/3	0/3	2/4	$10^{-6.5}$	.3
	$10^{-7.0}$	0/4		0/3	0/3	"	"	0/4		
	$10^{-7.5}$	1/4		"	"	"	"	"		
	$10^{-8.0}$	0/4		"	"	"	"	"		
	$10^{-8.5}$	"								

* Indicates the 10% mouse lung suspension inoc. intra-allantoically into 3 chick embryos.
† Numerator denotes No. infected; denominator denotes total No. per group.
‡ $E.I._{50}$ = 50% egg infectivity titer.
§ $M.I._{50}$ = " mouse " " .

infectious unit as measured in mice and chick embryos was relatively constant for mouse-adapted and chick embryo-adapted influenza viruses several strains of each were employed. The results are summarized in Table III. It is strikingly clear that a marked distinction exists in every instance between unadapted and adapted viruses in relation to the comparative quantities of each essential to induce infection in a mouse. Whereas, with unadapted viruses approximately 80 to 270 times more virus was present in a mouse infectious unit than in an egg infectious unit, with 3 of the mouse-adapted strains approximately the same quantity of virus composed the infectious unit for either host. Even greater contrast was noted with mouse-adapted Rhodes virus which manifested the capacity to infect the mouse with one-tenth the quantity of this agent required to produce infection in the embryonated egg. The marked discrepancy between infectivity titers of chick embryo-adapted viruses and the similarity of titers of mouse-adapted viruses as determined in both hosts cannot be attributed to the quantity of virus in the infected allantoic fluid tested. It will be noted in Table III that the chick embryo infectivity titer of viruses, unadapted or adapted to the mouse, varied markedly but the quantitative relationship described was maintained.

Discussion. The experimental evidence presented indicates that strains of influenza A virus which have been cultivated only in chick embryos required approximately 100 times more virus to infect mice than chick embryos. The ratio of egg infectivity titer to mouse infectivity titer (E:M) was of the same order of magnitude, *i.e.*, 2.0 log units for each of the strains tested. In marked contrast, influenza A and B viruses adapted to the mouse lung infected each host with approximately the same quantity of virus; the E:M infectivity ratio approached unity in 3 out of 4 examples. With the Rhodes strain of influenza virus the chick embryo was a less sensitive host than the mouse.

The relatively constant E:M infectivity ratio of egg-adapted viruses and probably a relatively constant but different ratio for mouse-adapted agents allow members of each group to be compared with others of similar host-adaptation characteristics by the use of infectivity dosages determined in embryonated eggs. In contrast, comparison in mice of unadapted with adapted strains in experiments in which the infectious dose of each agent is determined solely by titrations in the chick

TABLE III. Comparative Chick Embryo and Mouse Infectivity Titers Obtained by Parallel Titrations with Several Mouse and Chick Embryo Adapted Strains of Influenza A and B Viruses.

Virus titered	Mouse adapted	Infectivity titer— Chick embryo, $E.I._{.50}$	Mouse, $M.I._{.50}$	Ratio $E.I._{.50}/M.I._{.50}$, log
IAV—WRU 51-51	No*	$10^{-8.1}$	$10^{-5.6}$	2.5
— " " "–M	Yes	$10^{-6.8}$	$10^{-6.5}$	.3
—Rhodes	No	$10^{-8.1}$†	$10^{-6.5}$†	1.6
— " –M	Yes	$10^{-7.1}$†	$10^{-8.1}$†	–1.0
—CAM	No	$10^{-8.3}$	$10^{-6.0}$	2.3
—WRU 52-51	"	$10^{-6.7}$	$10^{-4.7}$	2.0
— " 53-51	"	$10^{-6.4}$	$10^{-4.6}$	1.8
—PR8	Yes	$10^{-8.2}$	$10^{-8.7}$	– .5
IBV—Lee	"	$10^{-6.0}$	$10^{-5.8}$	.2

* Chick embryo-adapted. † Geometric mean of results of 2 experiments.

embryo is probably a hazardous technic. Rather the more accurate but cumbersome procedure of combination mouse-egg titration should probably be employed.

Data have been presented by Friedewald and Pickels to indicate the extreme sensitivity of chick embryos to infection with exceedingly small quantities of egg-adapted influenza virus(9). The results of the experiments reported suggest that with properly adapted influenza virus the mouse is an equally sensitive host to infection with this agent. Infection of mouse lungs with relatively small amounts of adapted strains is even more surprising when one considers the relative inefficiency of intranasal inoculation of mice. For example, with PR8 virus it was estimated that of the order of only 18% of the inoculated virus actually initiated progressive infection in the mouse lung(10).

The factors which permit an agent to adopt the cellular environment of a new host for its biological activities are not clear. Since unadapted viruses multiply extensively in the mouse lung(11), adaptation of an influenza virus to this organ implies the acquisition by that agent of the property to inflict cell damage which results in pulmonary consolidation. Certain characteristics have been ascribed to these mouse-adapted viruses which distinguish them from unadapted strains(1-3). That mice can be infected with considerably less adapted than unadapted virus suggests at least a partial explanation for some of the previously described multiplication characteristics(1,2) of mouse-adapted influenza virus. In addition, this finding may be considered a further biological property of the mouse-adapted agent.

Summary. Parallel titrations to determine the dilution of virus essential to initiate infection in the allantoic membrane of the embryonated egg and the mouse lung were carried out. In every instance with mouse-adapted viruses, the relative quantity essential to infect the mouse and the chick embryo was approximately the same; with egg-adapted viruses approximately 2 logs more virus was required to initiate multiplication in the mouse lung than in the allantoic sac. These data describe another property of mouse-adapted influenza viruses which distinguish them from unadapted strains.

The excellent technical assistance of Miss Mary K. Dixon was of inestimable value in this investigation.

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ACTH Secreting Transplantable Pituitary Tumors.*† (20607)

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Pituitary tumors can be induced by ionizing irradiation(1). In order to find out whether these tumors are hormone-secreting and if so, to ascertain the type of hormones they secrete, attempts were made to transplant them in mice of the strain in which the tumor originated (LAF_1). Furthermore, it was thought possible that these tumors, like those induced by I^{131}, are conditioned neoplasms(2), and that the necessary conditioning procedures(3) might yield information as to the pathogenesis of pituitary tumors induced by ionizing irradiation. Accordingly, recipients were subjected to gonadectomy or radiothyroidectomy or X irradiation before grafting on them the tumors induced by ionizing irradiation.

The first attempt to graft a pituitary tumor occurring among mice exposed to ionizing irradiation yielded a transplantable growth which, contrary to expectations, proved autonomous, that is, transplantable to normal mice as well as to those variously pretreated (conditioned). The salient changes produced in the recipients were: obesity, with atrophy of the thymus and spleen, polyuria sometimes with glycosuria, hyperglycemia, and leukopenia, notably lymphocytopenia. These findings constitute sufficient presumptive evidence that the tumors secrete ACTH. Correspondingly, the adrenal glands of mice bearing these grafted tumors were hyperplastic while the gonads, thyroid, and other organs examined gave no indication of stimulation by other types of pituitary hormones. The following is supporting evidence:

Transplantation data. The original tumor (O.G. 1) was grafted in the thigh muscle of 23 mice of the strain of origin, of which all but 2 exhibited most, or all, of the changes stated above. Four males of this group were castrated 5 days after the tumor graft; 4 sibling males were non-castrated. Three females received 250 μc of I^{131} 3 days after the tumor graft; 3 female siblings remained untreated. Nine females received 200 r of 250 kvp X rays approximately 1 year before the tumor graft. The period of latency of tumor development as ascertained by palpation varied from 110 to 207 days. Following identification of the tumor, the hosts lived for periods of several weeks; most of them were sacrificed. At death the tumor measured a few mm to 10 mm across. Two subpassages were made with success as indicated by leukopenia or polyuria, after a somewhat shortened period of latency in all 16 mice that were grafted with tumor fragments.

Polyuria and glycosuria. The urinary volume of normal mice placed in a metabolism cage averages about 1-2 cc per day. The urinary volumes of mice bearing grafted tumors observed in the metabolism cage for at least 1 week varied between approximately 20 to 30 cc/day. The water intake was correspondingly increased. Of 15 mice with polyuria 2 had severe glycosuria. None of the numerous uninjected controls studied gave significant quantities of sugar in the urine.

Hyperglycemia. Blood sugar determinations were made on 8 tumor-bearing mice with leukopenia or polyuria. The animals were fasted for 16 hours. Samples of 0.05 ml of blood were obtained from the tail vein of heparinized mice and analyzed by the Folin-Wu method, slightly modified. Heparin had no effect on blood sugar levels. An approximately double number of normal mice were tested simultaneously with tumor-bearing mice in each experiment. The values for reducing sugars in mg per 100 ml blood were as follows:

Blood sugar, mg %	
Normal mice†	Mice with tumor grafts*
88	104
115, 120	160, 200, 230
150, 160	170, 208, 210, 330

* Individual values on animals with polyuria and leukopenia.

† Mean values of 2-3 mice.

* Work performed under contract for the U. S. Atomic Energy Commission.

† Drs. H. B. Andervont, W. E. Heston, and V. P. Bond kindly provided us with normal mice of the LAF_1 strain used in these studies.

The high normal values may be due to inadequate fasting of mice in the respective experiments.

White cell counts. The white cell count of 9 tumor-bearing mice ranged from 300 to 3,150, averaging 1,150. The % of lymphocytes ranged from 4 to 44, averaging 15. The control white cell counts made simultaneously on 3 normal animals varied from 9,650 to 12,050 with an average of 11,233. The lymphocytes averaged 84.7%.

Obesity. Every tumor-bearing animal was either moderately or highly obese.

Microscopic observations. The adrenals of all tumor-bearing mice were slightly to greatly enlarged. The cells of all 3 layers of the cortex were swollen. The tumor cells were fairly uniform in size and shape. The nucleus was nearly normal in size and appearance; the cytoplasm was abundant and all cells contained in their cytoplasm numerous minute granules which were distinctly stained with hematoxylin. None of the other endocrines exhibited noteworthy changes. Detailed description and illustration of the tumors, adrenals, and other organs will follow.

Surgical removal of the adrenal was successful in one tumor-bearing mouse in which the 24-hour urinary volume, 20 cc before operation, dropped to 2 cc after the operation and remained within the normal range until death, 24 days after operation.

Comments. Thirteen pituitary tumors induced by radiothyroidectomy in 3 strains of mice were transplanted in series to a total of well over 1000 mice. Transplantation of these tumors succeeded at first only in radiothyroidectomized and not in normal mice. All secreted thyrotrophin and none ACTH. In contrast, the tumors which originated in a mouse exposed to ionizing irradiation and analyzed in this study produce secondary changes indicative of ACTH secretion. A second tumor of similar provenance currently analyzed is identical in every respect with that here described. The evidence presented elsewhere(1) strongly suggests that the tumors occurring among mice exposed to ionizing irradiation are actually induced by the latter either by a direct or indirect action, their incidence among controls 25 months after exposure being 0.15% as compared to 4 to 12% in groups receiving about 200-700 r. The present study indicates that these tumors might be related to the original insult of the adrenal-pituitary mechanism caused by a single exposure of gamma rays or neutrons.

Excessive doses of cortisone produce in mice lymphopenia, loss of body weight, atrophy of thymus and spleen(4). The animals in our experiment did not lose and usually gained weight. This suggests that obesity and gain in weight are due to hormones other than cortisone produced in excess (directly or indirectly) by the grafted pituitary tumors.

Summary and conclusions. Two pituitary tumors occurring in mice exposed to ionizing irradiation proved readily transplantable causing in the recipients obesity with thymic atrophy, hyperglycemia, polyuria, sometimes with glycosuria, severe lymphocytopenia, and hypertrophy of the adrenal cortex. On the basis of these findings it is concluded that this tumor secretes ACTH. There is lack of evidence of stimulation of any endocrine organ other than the adrenal.

The able assistance of Miss Bonnie Anderson and Mr. Charles Gurney is hereby acknowledged.

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Induction of Pituitary Tumors by Means of Ionizing Irradiation.*† (20608)

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The occurrence of pituitary tumors among mice exposed to X rays has been mentioned before(1,2), but no evidence has been presented thus far to indicate that these tumors were actually induced by ionizing irradiation. In all probability earlier workers did not examine the cranial cavity systematically, or the experimental animals were not observed long enough after irradiation.

Methods. Large numbers of mice of the LAF_1 strain were exposed to atomic detonation. Some were not shielded and received predominantly gamma rays of high energy; some were lead-shielded to eliminate most of the gamma rays. The latter group will be spoken of as neutron-exposed animals. A fuller account of the exposure conditions and energy calculations will be given later. Approximately 30 days following exposure the surviving mice were transported to Oak Ridge where they remained individually caged until natural death or were killed in extremis. All animals have been autopsied and are preserved. The tumor diagnosis, as tabulated, is based on gross examination confirmed by microscopic examinations. At autopsy most tumors replaced the anterior hypophysis completely and compressed the overlying brain. In animals bearing large tumors the posterior part of the skull was bulging. Numerous animals in which the clinical diagnosis was made on this basis, were sacrificed and the bulk of the tumors was used in transplantation studies(3). On microscopic examination the tumors appeared chromophobe usually with many cavernous vascular spaces. Invasion of the sella and its marrow cavity was frequent even though the cells appeared uniform, lacked hyperchromatophilia and nuclear abnormalities indicative of malignancy. A detailed description of the gross and microscopic features of these tumors will follow. Minute tumor nodules discovered on microscopic examination have not been included in the tabulation. Hemorrhage in the tumor or compression of the brain appeared to be the cause of death in most instances.

Results. Table I indicates that 4 to 12% of the females exposed to 192-733 r developed pituitary tumors as compared to 0.3% of the controls. No pituitary tumor occurred among the control males during this period of observation while 2 to 5% of the males exposed to 491-785 r developed such tumors. The dose acutely fatal to 50% of the animals was about 750 r. Most mice exposed to larger doses died within 10-14 months, and this probably accounts for the absence of pituitary tumors in mice exposed to higher doses, since, as Table II indicates, the period of latency of these tumors is very long.

Table II shows that pituitary tumors began to appear in larger numbers 16 months post-irradiation, and they occurred with greatest frequency in the 23-25 months postirradiation groups. It is well known that chromophobe adenomas of the pituitary gland are common among old rats, but very little is known about pituitary tumors in mice.

That pituitary tumor-induction dose is low is indicated in Table III which lists the findings on neutron-exposed mice. It seems that a dose as low as 40 to 70 rem may induce pituitary tumors. Further work is required to establish the relatively greater efficiency of neutron as compared with gamma irradiation in the induction of these neoplasms, suggested by Table III.

These findings, representing only a part of a very large scale experiment, are released here in a preliminary form because:

(a) They indicate that ionizing irradiation can produce pituitary tumors in at least one strain of mice, and this knowledge might induce other investigators to a closer study of this organ in mice of other strains and of animals of different species exposed to ioniz-

* Work performed under contract for the U. S. Atomic Energy Commission.

† The faithful assistance of Peggy Ledford, Frances Farbstein, William Gude, and numerous animal caretakers is gratefully acknowledged.

TABLE I. Longevity and Incidence of Pituitary Tumors in Mice Exposed to Atomic Detonation.

Dose (r)*	No. exposed,† ♂ and ♀	Female mice surviving‡ 30 days No.	%	1 yr No.	%	25 mo No.	%	Pituitary tumor No.	%	Male mice surviving* 30 days No.	%	1 yr No.	%	25 mo No.	%	Pituitary tumor No.	%
812-932	880	25	6	15	3	0	0	0	0	39	9	25	6	0	0	0	0
759-785	440	87	40	57	26	0	0	0	0	85	39	58	26	4	2	3	5
711-733	440	157	71	118	53	0	0	14	12	153	70	130	59	13	6	2	2
631-687	440	195	89	146	66	9	4	17	12	204	93	176	80	37	17	8	4
491-556	440	215	98	179	81	43	19	22	12	208	95	185	84	75	34	4	2
367-424	440	78§	—	66	—	23	—	7	11	108§	—	102	—	43	—	0	0
287-318	440	213	97	195	89	58	26	10	5	213	97	202	92	112	51	2	1
192	220	107	97	102	93	45	41	4	4	107	97	101	92	62	56	0	0
0	620	301	97	300	97	189	61	1	0.3	310	100	294	95	198	64	0	0

* Predominantly (over 90%) gamma radiation; the ratio of gamma rays to neutrons being somewhat greater in the lower dose range.
† Half males, half females.
‡ Post-irradiation. The mice were irradiated at 6-12 wk of age. This tabulation was prepared approximately 26 months post-irradiation.
§ These figures are incomplete due to loss of animals during transportation from field laboratories.

TABLE II. Incidence of Pituitary Tumors in Mice Exposed to Atomic Detonation.

Mo post-irradiation	Female No. dying	Female Pituitary tumor No.	Female %	Male No. dying	Male Pituitary tumor No.	Male %
1-10	139	0	0	115	0	0
11-13	83	3	4	56	0	0
14-16	148	3	2	81	0	0
17-19	244	22	9	178	6	3
20-22	231	21	9	241	7	3
23-25	215	26	12	226	4	2

TABLE III. Incidence of Pituitary Tumors in Female Mice Exposed to Neutron Radiation from Atomic Detonation.

Dose* (rem)	No. exposed	Mice surviving 30 days No.	30 days %	1yr No.	25mo No.	Pituitary tumor No.	Pituitary tumor %
450	30	27	90	20	4	2	10
180	30	28	93	22	3	3	14
140	20	20	100	18	2	3	17
70	30	30	100	29	14	2	7
40	30	27	90	24	13	1	4
0	310	301	97	300	189	1	0.3

* The rem values were calculated on the basis of thymic wt loss following 230 kvp X radiation by Dr. R. E. Carter and associates.

ing irradiation.

(b) They indicate the long period of latency required for the development of these tumors, and this knowledge might induce others not to destroy irradiated animals prematurely.

(c) These data present the background to the transplantation studies of pituitary tumors described currently(3), indicating that the first 2 of these tumors successfully grafted on normal mice of this strain secrete ACTH.

(d) It is possible that these tumors are induced not by a direct action of ionizing irradiation on the pituitary but by some indirect mechanism, the elucidation of which calls for a renewed study of the effects of irradiation on endocrine and other organs under pituitary control.

It is generally believed that female mice live longer than males. The present data show the reverse, and this may be due to several factors, such as, strain differences, individual caging of the animals which prevented fighting among the males, or greater incidence of neoplasms and other diseases among females, at an earlier age.

Summary and conclusions. Pituitary tu-

mors were induced in mice by a single exposure to ionizing irradiation. The greatest incidence occurred among mice exposed to doses of gamma radiation somewhat below the LD_{50}. These tumors were about four times more frequent among females than among males. Neutrons appear more efficient in inducing these neoplasms than gamma rays, and the data suggest that doses as low as 40-70 rem may induce such tumors. The period of latency of these neoplasms is very long, the maximum incidence being at 17-25 months postirradiation.

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